INTEGRATED MASTER'S IN BIOENGINEERING
SPECIALIZATION IN BIOLOGICAL ENGINEERING

Studies of sesquiterpene lactones with potential antitumoral activity: purification, synthesis of aminoderivatives and cytotoxicity assays

Master Thesis

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Abstract

The present work aimed at the implementation of a semi-preparative high-performance liquid chromatography (HPLC) method that allows for the separation of two guaianolide-type STLs (compounds **6** and **7**) from a mixture of both compounds. Moreover, the work comprised the semi-synthesis of new aminoderivatives of these compounds with improved bioavailability, due to increased water-solubility and lower toxicity while retaining their anti-tumoral activity. Alongside, a preliminary screening has been conducted to assess the potential cytotoxicity of one of the STLs (compound **3**) towards normal, non-neoplastic cells, specifically fibroblasts.

The complete semi-preparative separation of the two STLs (**6** and **7**) was not accomplished, even though different mobile phase conditions were tested. A high load (100 µL) sample of the mixture of **6** and **7** onto the C18 column resulted into a decrease in chromatographic resolution due to peak width broadening, which led to a significant co-elution of **6** and **7**, with retention times of 38.81 min and 39.89 min, respectively.

Furthermore, *N, N*-dimethylaminogrosshemin-2,3-dihydroxyisobutyrate (**6a**) and *N, N*-dimethylaminodehydrogrosshemin-2,3-dihydroxyisobutyrate (**7a**) were obtained through a Michael addition of dimethylamine to the α-methylene-γ-lactone ring, more specifically to the electrophilic vinylic carbon C-13, of **6** and **7**, respectively. The semi-synthesis of the aminoderivatives was confirmed through comparison of their ^{13}C NMR and ^{1}H NMR spectra with the complete NMR characterization of STLs **6** and **7**.

Finally, the preliminary *in vitro* cytotoxicity studies of compound **6** on normal human fibroblasts indicated, through observation of the cells on an inverted fluorescence microscope and percent viability of cells data, that STL **6** is not toxic towards normal human foreskin fibroblast cells in concentrations of up to 2.75 µM after a period of incubation from 24h – 72h.

Keywords: sesquiterpene lactones, aminoderivatives, HPLC-DAD, NMR, cytotoxicity.

Contents

List of Figures

List of Tables

Glossary

ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
BVOC	Biogenic Volatile Organic Compound
COSY	Correlation Spectroscopy
DAD	Diode Array Detection
DEPT	Distortionless Enhancement by Polarization Transfer
DMAPT	Dimethylaminoparthenolide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
FPP	Farnesyl Diphosphate
GC	Gas Chromatography
HFF	Human Foreskin Fibroblast
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence
iNOS	Inducible Nitric Oxide Synthase
MDM2	Mouse Double Minute 2 Homolog
MEKC	Micellar Electrokinetic Chromatography
MS	Mass Spectrometry
NCI	National Cancer Institute
NF-κB	Nuclear Factor κB
NMR	Nuclear Magnetic Resonance
OPLC	Overlay Pressure Liquid Chromatography
PBS	Phosphate Buffered Saline

PTL	Parthenolide
RP	Reversed Phase
SERCA	Sarco/Endoplasmic Reticulum Calcium ATPase
SFC	Supercritical Fluid Chromatography
STL	Sesquiterpene Lactone
TLC	Thin Layer Chromatography
UV	Ultraviolet
αMγL	α-methylene-γ-lactone

1. Introduction

1.1. Review on sesquiterpene lactones

In 1960, as part of an effort to discover naturally occurring anticancer agents, the National Cancer Institute (NCI, USA) launched a large-scale screening program where 35 000 plant samples were evaluated, from which arose Taxol, the best-selling antitumor drug nowadays (Ghantous et al. 2010). Actually, over the last half century most plant secondary metabolites and their derivatives have been applied towards the treatment of cancer, which totalizes 67% of all anticancer drugs. Furthermore, over 200 natural product-derived drugs are in preclinical or clinical development (Balunas & Kinghorn 2005; Ghantous et al. 2010; Harvey 2008). Research on the natural compounds sesquiterpene lactones (STLs) began within few years of the NCI screening program leading to the identification of STLs as one of the most prevalent groups of cytotoxic and anti-tumoral compounds from plants (Fernandes et al. 2008; Ghantous et al. 2010).

Sesquiterpene lactones are one of the largest groups of secondary metabolites with biological significance that are found in several species across the plant kingdom, belonging predominantly to the Asteraceae (or Compositae) family, where they can be found at concentrations varying from 0.001% to 8% of plant dry weight (Ghantous et al. 2010; Amorim et al. 2013; Chadwick et al. 2013). In its turn, Asteraceae is one of the largest family of flowering plants (angiosperms), with over 23 000 currently recognized species spread across 1 620 genera and 12 subfamilies being distributed worldwide, occurring in every continent except Antarctica and prospering in any habitat compatible with the development of vascularity (Bohm & Stuessy 2001; Cronquist 2001; Amorim et al. 2013).

STLs are typically located in laticifers, which are plant specialized cells that have a secretory nature (Chadwick et al. 2013). Laticifers serve as a repository for natural products, including proteins and other metabolites present in latex, which have been shown to protect plants against feeding insects, herbivores and that are also beneficial for humans (Hagel et al. 2008).

In fact, in most of the Asteraceae, STLs act as communication substances that help organisms to cope with each other and as a defence mechanism against predation (Amorim et al. 2013). These compounds are emitted, among a wide range

of different compounds, by plants during the biogenic volatile organic compound (BVOC) response (Chadwick et al. 2013), although they constitute a low percentage of BVOCs due to their low volatility (Tschiggerl & Bucar 2012). BVOCs not only constitute a platform for plant-plant interactions, prompting nearby plants' defence mechanisms against insects and other predators, but can also be toxic to insects. Their potential as anti-feedants is also due to their bitterness which repels chewing insects and birds that disrupt cells when feeding (Chadwick et al. 2013; Kegge & Pierik 2010).

Allelopathic function is also verified in sesquiterpene lactones exuded into the rhizosphere (Chadwick et al. 2013) - a biologically active and densely populated soil zone (Walker et al. 2003) - through the roots, in the form of BVOCs. This involves offensive strategies that inhibit the performance of proximate competitors, for instance many sesquiterpene lactones from sunflowers reduce the germination rates of plants from other families (like Solanaceae) to reduce competition in the area (Chadwick et al. 2013; Kegge & Pierik 2010).

Likewise, there is evidence of an antimicrobial function in which the polar groups present in STLs disrupt the phospholipid membrane of microorganisms preventing their attack (Chadwick et al. 2013).

Human populations are mainly exposed to STLs through their use as herbal remedies, as part of a western diet – STLs of the guaianolide class, such as lactucin, lactucopicrin, and 8-deoxylactucopicrin are found in lettuce (*Lactuca sativa*) (Chadwick et al. 2013) – and as therapeutic drugs (Amorim et al. 2013). However, exposure to STLs in their natural habitats can cause adverse effects due to direct contact with plants rich in STL allergens, such as parthenin, helenalin, helenin and hymenin, which are manifested as allergic contact dermatitis (Ghantous et al. 2010; Salapovic et al. 2013).

The main interest in sesquiterpene lactones for health benefit purposes is related to their anti-tumoral potential, since some of the STLs have been found to show enough potential to enter clinical trials. Nevertheless, there is a wide variety of other pharmacological applications of STLs, including anti-inflammatory, anti-bacterial, anti-fungal, anti-viral, anti-protozoal, anti-helminthic, anti-ulcer, molluscicidal, hepatoprotective and hepatocurative, anti-depressant, anti-malarial, anti-migraine, analgesic and sedative. They are also considered in the treatment of cardiovascular diseases, prevention of neurodegeneration, and treatment of mild illnesses such as diarrhoea, flu, and burns. The cardiovascular effects are attributed

to their ability to relax smooth muscle tissue by inhibiting the nuclear factor-kB (NF-κB), which in its turn inhibits the inducible form of nitric oxide synthase (iNOS) up-regulation, and consequently increases levels of NO (Amorim et al. 2013; Chadwick et al. 2013; Ghantous et al. 2010).

STLs are a colourless, bitter and stable subfamily of terpenoids of lipophilic character composed of a 15-carbons (C_{15}) backbone that contains a lactone group (Ghantous et al. 2010). There were over 5000 different members of the STL family in 2000, a number which continued growing since then, as covered in the reviews on natural sesquiterpenoids by Fraga every year (Fraga 2013). Thus, they are very diverse in terms of their structure, properties, and proposed functions (Chadwick et al. 2013). Generally, considering their carbocyclic skeleton, they can be divided into the following major groups, which are generally represented in Figure 1: germacranolides (**a)** (ten-membered ring); eudesmanolides (**b)** and eremophilanolides (**c**) (all 6/6-bicyclic compounds); and guaianolides (**d**) and pseudoguaianolides (**e**) (all 5/7-bicyclic compounds) (Amorim et al. 2013; Ghantous et al. 2010). All STLs are derived from farnesyl diphosphate (FPP) through cyclization and oxidative transformation yielding, for instance, costunolide (**1**), the common precursor for germacranolides, eudesmanolides and guaianolides (Eljounaidi et al. 2014). Furthermore, they all contain a fused 5-membered lactone group (γ-lactone) and they mostly have one methylene group at the alpha position to the carbonyl group (Fraga 2013).

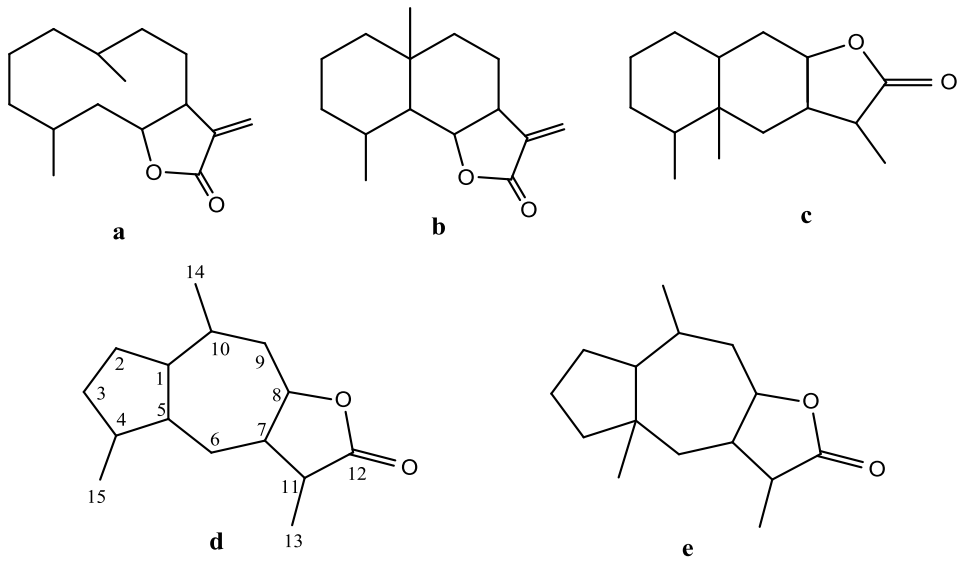

Figure 1. Chemical structures of different subgroups of sesquiterpene lactones. (a) Germacranolide; (b) eudesmanolide; (c) eremophilanolide; (d) guaianolide; (e) pseudoguaianolide.

STLs' biological activity is mainly due to an alkylation mechanism by which they covalently bond with biological macromolecules. Thus, alkylating centre reactivity influences their activity, with the major alkylating centres being the α-methylene-γ-lactone, α-methylene-δ-lactone, δ,β-cyclopentenones and side chain unsaturated esters (Amorim et al. 2013; Ghantous et al. 2010).

The α-methylene-γ-lactone group (αMγL), an oxygen-containing ring structure with a carbonyl function, is viewed as essential for STLs' biological effects, with other functional groups in the structure merely modifying the ring's potency by steric and chemical influences. Moreover, the αMγL unit exerts its therapeutic influence by means of alkylation of thiol groups commonly found in its molecular targets. It reacts with biological nucleophiles by Michael-type addition of free cysteine sulphydril (-SH) groups (typically residues in proteins), therefore, forming stable adducts (Figure 2). Consequently, they cause alteration of gene expression, by inhibiting enzymes involved in biological processes, with the effect of sensitizing cancer (Chadwick et al. 2013; Fernandes et al. 2008; Foster et al. 2011; Rivero et al. 2003; Scotti et al. 2007).

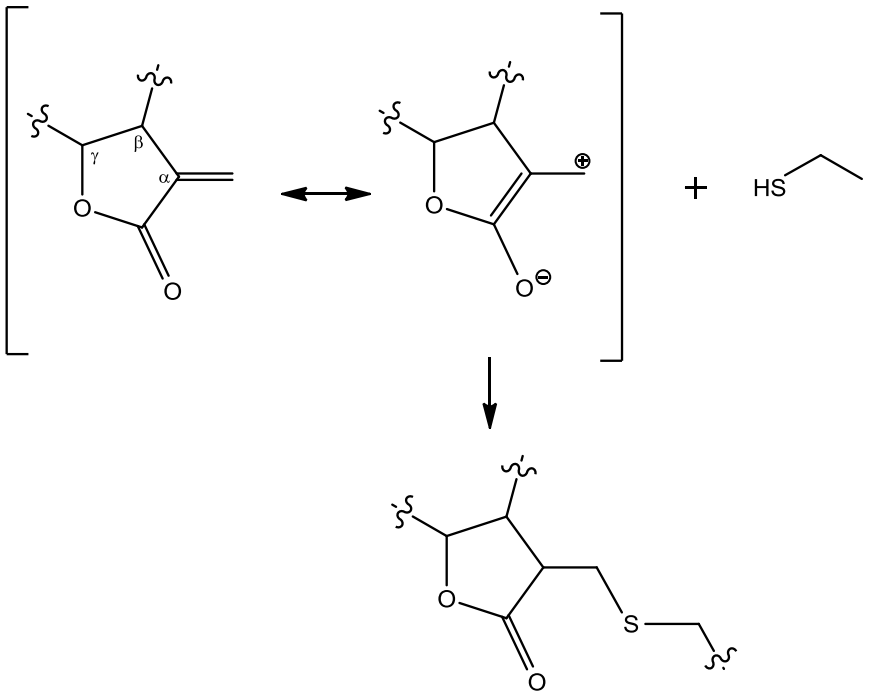

Figure 2. Michael addition of thiols to the α-methylene-γ-lactone of sesquiterpene lactones.

However, the unspecific, non-selective nature of this action causes STLs to bind to blood proteins containing sulfhydryl groups, reducing their bioavailability and

preventing them from achieving concentrations that are crucial to cytotoxicity (Amorim et al. 2013; Ghantous et al. 2010).

The presence of a side chain and/or the level of lipophilicity can also affect the activity of these compounds. On the one hand, higher lipophilicity may facilitate penetration through the cytoplasmic membrane, by this means increasing STLs cytotoxicity *in vitro,* causing them, in return, to be less bioavailable *in vivo*. On the other hand, a side chain ester, in compounds having the α-methylene-γ-lactone as the only alkylating centre, increases cytotoxicity regardless of its lipophilic properties although in the presence of two alkylating centres, an ester side chain has to be lipophilic and conjugated to increase STL power. Moreover, it was verified that the presence, in an proper site, of an OH group enhanced STL bioactivity while decreasing lipophilicity (Ghantous et al. 2010).

Molecular geometry and electronic features also plays an important role in STL cytotoxicity. The guaianolides and the pseudoguaianolides are the most active compounds owing to the double bond in the five-membered ring at C-3, as well as to a methylene at C-10, present in the most potent STLs within these two families (Fernandes et al. 2008; Ghantous et al. 2010).

STLs biochemical mechanisms for selectively targeting cancer cells have been object of systematic study for several years now. As a result several of these compounds, including a parthenolide (PTL, **2**) derivative – dimethylamino-parthenolide (DMAPT, **2a**) –, two artemisinin derivatives – artesunate (**3**) and artemether (**4**) – and thapsigargin (**5**), are now in cancer clinical trials (Table 1; Figure 3). Their selectivity to target tumor and cancer stem cells while sparing normal ones is attributed to their ability to target the sarco/endoplasmic reticulum calcium ATPase (SERCA) pump, particular proteases secreted by cancer cells, high iron content and cell surface transferrin receptors, NF-kB signaling pathway, protein MDM2 degradation and tumor suppressor/protein p53 activation, and to interfere with angiogenesis, metastasis and epigenetic mechanisms (Amorim et al. 2013; Ghantous et al. 2013).

A significant amount of research on cancer treatment has been aimed at the NF-kB pathway. NF-κB is a protein consisting of a p50 and p65 subunit and, when released from the IκB subunit, acts as a transcription factor on several genes in immune responses, inflammation pathways and apoptosis pathways and metastasis, by controlling response of other effectors such as cytokines, inflammatory molecules and cell adhesion molecules. Consequently, inhibition of the NF-κB complex,

specifically inhibition of its release from the IκB complex, reduces inflammatory response and prevents cancer growth (Amorim et al. 2013; Chadwick et al. 2013).

Table 1. Sesquiterpene lactones in cancer clinical trials.

Compound	Group of STL	Plant species	Cancer type	Clinical trial	Ref
DMAPT (2a)	Germacranolide	*Tanacetum parthenium*	Acute myeloid leukemia (AML) Acute lymphoblastic leukemia (ALL)	Phase I	(Ghantous et al. 2010)
Artesunate (3)	Cadinanolide	*Artemisia annua* L.	Breast and colorectal cancer Non-small cell lung cancer Uveal melanoma Laryngeal carcinoma	Phase II	(Berger et al. 2005; Efferth et al. 2001; Ghantous et al. 2010; Singh & Verma 2002)
Artemether (4)	Cadinanolide	*Artemisia annua* L.	Pituitary macroadenoma	Phase II	(Ghantous et al. 2010; Singh & Panwar 2006)
Thapsigargin (5)	Guaianolide	*Thapsia garganica* L.	Breast, kidney and prostate tumors	Phase I	(Ghantous et al. 2010)

The germacranolide parthenolide (**2**), [4α, 5β-epoxy-germacra-1-(10),11-(13)-dien-12,6α-olide] – an epoxy-costunolide – is a recognized NF-kB inhibitor. It has been isolated from several different species in the Asteraceae family, being the major active principle of *Tanacetum parthenium*. In the past few years, an increasing interest has been observed in this sesquiterpene lactone, due to its significant antitumor activity. The latter is a result of its strong inhibition of NF-kB, by targeting multiple steps along the NF-kB signaling pathway, including the modification of the NF-kB p65 subunit and suppressing the activity of the upstream IkB kinase complex leading to the stabilization of the NF-kB inhibitors, IkBa and IkBb. The nucleophilic attack by parthenolide happens through the αMγL ring and epoxide moieties that target specific nucleophiles but not others (Ghantous et al. 2013; Ghantous et al. 2010; Nasim & Crooks 2008).

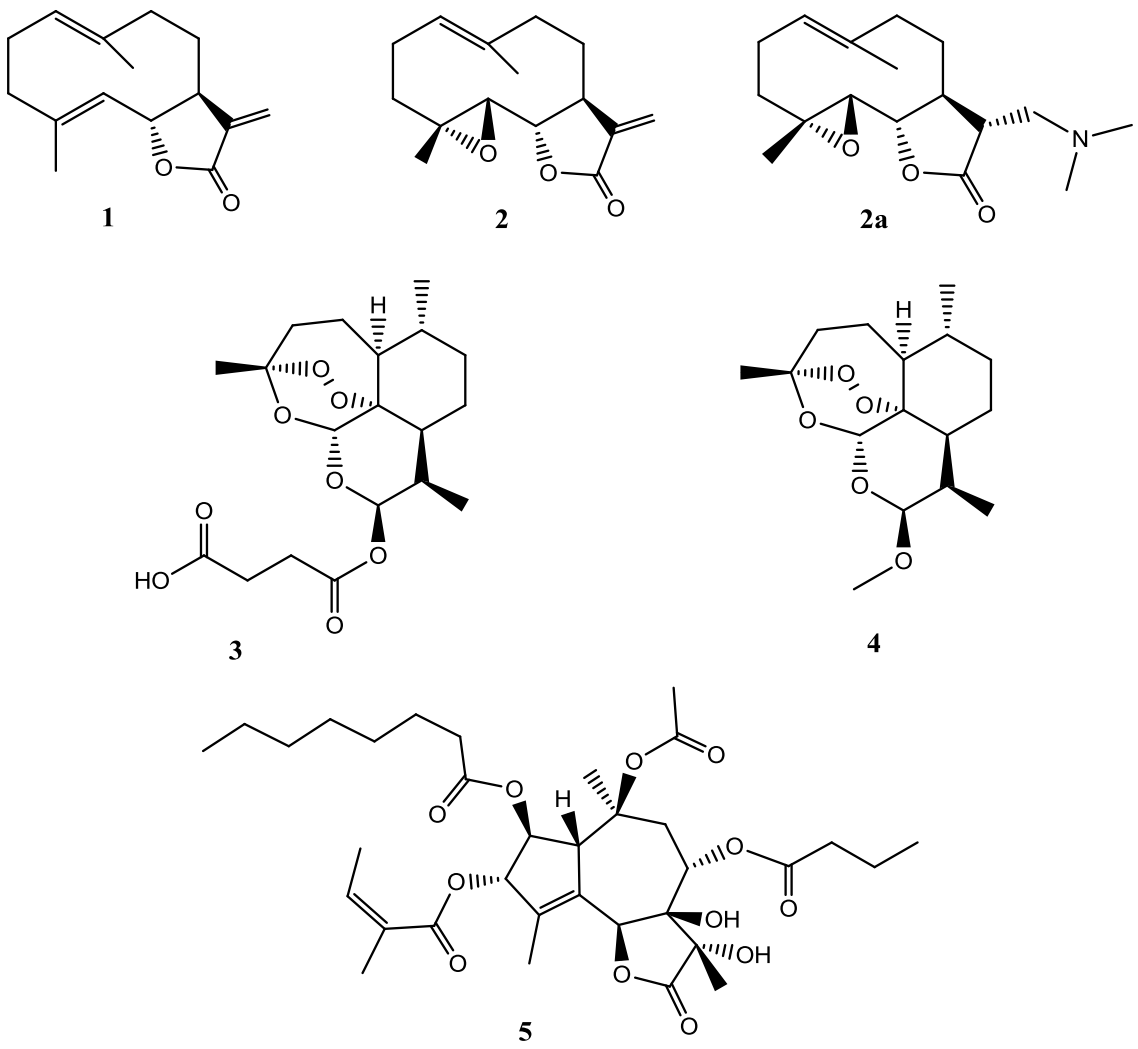

Figure 3. Structures of STLs in clinical trials and others related.

However, the low oral bioavailability of PTL, due to poor water-solubility (0.169 μM/mL maximum solubility in serum), and its indiscriminate reaction towards biological nucleophiles are a major limitation for its clinical use and development (Fardella et al. 1999; Ghantous et al. 2013; Nasim & Crooks 2008; Neelakantan et al. 2009).

Prompt by the above mentioned, new strategies consisting of derivatizing STLs into amino analogs have emerged, in which an amine is added into the αMγL of STLs to mask this group from nucleophiles and increase water solubility while retaining biological activity (Woods et al. 2013; Neelakantan et al. 2009).

Thus, the Michael addition of the secondary amine dimethylamine led to the synthesis of the more water-soluble and orally bioavailable analog of PTL, dimethylaminoparthenolide (DMAPT), as illustrated in Figure 4. In fact,

pharmacokinetic analysis of in vivo studies in the rat showed that DMAPT has an oral bioavailability of around 70%, being predominantly metabolized into a product of mono N-demethylation (Chadwick et al. 2013; Neelakantan et al. 2009).

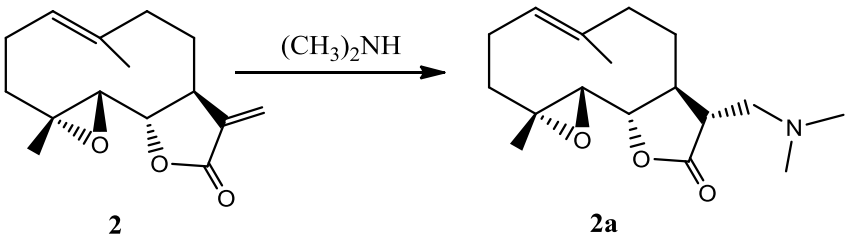

Figure 4. Semi-synthesis of DMAPT (2a) by Michael addition of dimethylamine to PTL (2).

DMAPT causes apoptosis by decreasing the binding of the Rel-A subunit of NF-κB to DNA, supressing the transcription of numerous NF-κB-regulated genes, associated with cancer cell survival and resistance to chemotherapy (Neelakantan et al. 2009).

1.2. Project presentation

Two new sesquiterpene lactones of the guaianolide type - grosshemin 2,3-dihydroxyisobutyrate (**6**) and dehydrogrosshemin-2,3-dihydroxyisobutyrate (**7**) – were previously isolated from *Centaurea ornata* (Bastos et al. 1994). Germacranolides, eudesmanolides, elemanolides and guaianolides are typically found in representatives of the genus *Centaurea* (Bruno et al. 2005).

Moreover, their structure has been elucidated by Nuclear Magnetic Resonance (NMR). The only structural differences among these guaianolides is a ketone group at C-3 and a methyl group (C-15), in (**6**), which is replaced by a hydroxyl group and a methylene group, respectively, in (**7**), as can be seen in Figure 5.

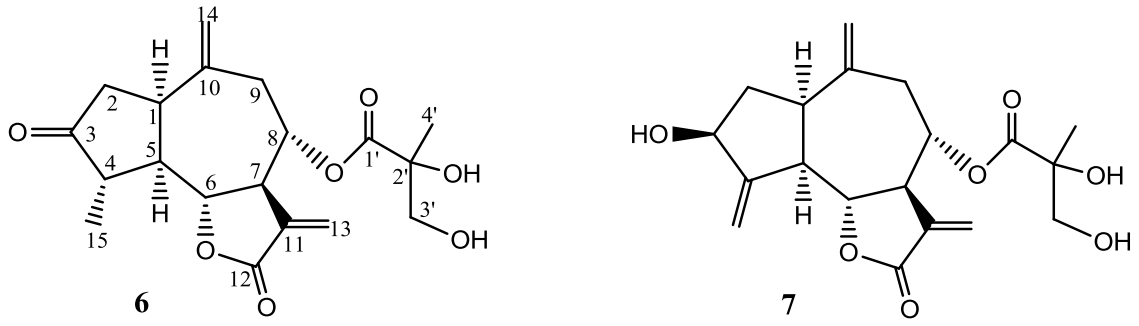

Figure 5. Structures of grosshemin 2,3-dihydroxyisobutyrate (6) and dehydrogrosshemin-2,3-dihydroxyisobutyrate (7).

STLs' anti-tumoral activity has been scientifically demonstrated by several research groups for different compounds. On the other hand, it is important to evaluate the toxic side-effects of these compounds, i.e. their safety. Thus, before specific studies of anti-tumoral activity of these two compounds are performed on cancer cell lines, a preliminary screening has been conducted, to assess the potential citotoxicity of compound **6** towards human fibroblasts, which are normal, non-neoplastic cells.

Once these compounds have been proven to possess anti-tumoral activity, and having isolated plant fractions which contain these STLs, the main challenge is the development and implementation of a method for their purification and separation from a mixture, as pure substances, using efficient preparative chromatographic separation techniques. In this sense, this study also aims at making use of semi-preparative high-performance liquid chromatography (HPLC) to develop a chromatographic method that enables the separation of compounds **6** and **7** from a mixture, obtained from different fractions of the plant.

Furthermore, in continuation of studies on the chemical modification of sesquiterpene lactones to produce new aminoderivatives with improved bioavailability, due to increased water-solubility and lower toxicity while retaining their anti-tumoral activity, new aminoderivatives based on the known and available grosshemin guaianolides (**6**) and (**7**) have been synthesized.

2. State of the art

2.1. High Performance Liquid Chromatography

In general, chromatographic methods for the separation in individual components, including high-performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC), gas chromatography (GC), thin layer chromatography (TLC), overlay pressure liquid chromatography (OPLC) and micellar electrokinetic chromatography (MEKC), are used to identify/purify new STLs. Furthermore, these methods are combined with analytical characterization techniques such as mass spectrometry (MS), diode array detection (DAD) and nuclear magnetic resonance (NMR), resulting in GC–MS, HPLC–DAD, HPLC–NMR or SFC–DAD, by which characterization and often identification of the separated compound is possible (Fritsche et al. 2002; Merfort 2002).

HPLC with ultraviolet detection (HPLC-UV) is often the preferred physical method of separation of these substances due to the low volatility and occasional thermolability of STLs (Merfort 2002). However, when sesquiterpene lactones have low molar absorption coefficient in the UV range, owing to weakly absorbing chromaphoric groups or no chromophores at all, HPLC-UV is not sensitive enough and, thus, unsuccessful for STL analysis (Merfort 2002; Yang et al. 2012).

Hence, the combination of HPLC with mass spectrometry (MS) has become extremely helpful in the detection and identification of STLs. In fact, it allows, not only the separation of the components of a mixture, but also the characterization of those components. Mass spectrometry can be used for obtaining information even when the compounds are not completely separated. The nature of MS detector allows the quantification of more than one compound even if co-eluted, as long as the ions have different mass/charge (Merfort 2002; Skoog et al. 2007).

Furthermore, high-performance separation techniques are often combined with a structurally elucidatory spectroscopic method, such as nuclear magnetic resonance (HPLC-NMR). NMR spectroscopy enables quantitative analysis, with practical application for mixture analysis and purity assessment due to an inherent characteristic in which integrated signal intensity (signal area) in the spectrum is proportional to the number of nuclei giving the corresponding resonance (Merfort 2002; Staneva et al. 2011).

On the other hand, efficient preparative chromatographic separation techniques are important for the rapid isolation of target compounds once their bioactivity has been confirmed. Essentially, while analytical chromatography does not depend on the recovery of a sample, preparative chromatography is a purification process employed with the purpose of isolating a pure substance from a mixture (Hostettmann et al. 1998).

The literature provides very few reports on the high-performance liquid chromatography using a semi-preparative column aiming at the separation and recuperation of sesquiterpene lactones contained in a plant fraction. Researches rather focus on the analytical separation, characterization and quantitative determination of these biologically active compounds. The HPLC methods for the separation of STLs by several authors are reviewed in Table 2.

It is noted that C18 is the most common silica-based stationary phase in the researches carried out in this field. Moreover, methanol-water is the preferred mobile phase, both in isocratic and gradient elution.

2.2. Semi-synthesis of aminoderivatives

The pharmacological benefit of sesquiterpene lactones has been extensively demonstrated and its biological effect has been attributed to the α-methylene-γ-lactone group. Nevertheless, their poor aqueous solubility and non-selective binding as a Michael acceptor at undesired targets renders unsuitable compounds for clinical development as a drug entity (Chadwick et al. 2013; Dell'Agli et al. 2009; Ghantous et al. 2010; Ghantous et al. 2013; Guzman et al. 2005; Guzman et al. 2007; Nasim & Crooks 2008; Neelakantan et al. 2009; Woods et al. 2013).

Therefore, a series of more water-soluble analogs have been synthesized. Ghantous et al. (2013) verified that reduction of the α-methylene, epoxidation of the endocyclic alkene or oxidation of the allylic methyl groups reduces activity of the derivatives. Thus, new studies were directed towards the synthesis of aminoderivatives.

Woods et al. (2013) described the semi-synthesis of serveral aminoderivatives of STLs, such as helenalin, ambrosin, costunolide and saussureamines A–E, alantolactone and parthenolide (PTL, **2**). Besides its chemical development, its pharmacological applications and potential as a clinical applicant is also addressed.

Table 2. Analytical and semi-preparative separations of sesquiterpene lactones by HPLC. [a]

Structure[b]	Column	Solvent[c]	Flow rate (ml/min)	Reference
		Analytical separation		
8,9	C18 (10 µm; 200x4.0 mm)	MeOH/H_2O (30:70)	0.8	(Fardella et al. 1999)
10	C18 (5 µm; 250x3.0 mm)	MeOH/H_2O var.	0.5	(Ferioli & D'Antuono 2012)
11	C18 (5 µm; 250x4.6 mm)	IPA/MeCN/MeOH/ H_2O/HCOOH var.	1.0	(Fritsche et al. 2002)
12	C18 (5µm; 250×4.6 mm)	MeCN/H_2O (55:45)	1.0	(Huo et al. 2010)
13	C18 (5 µm; 250x4.6 mm)	MeCN/H_2O,H_3PO_4 var.	1.0	(Sessa et al. 2000)
14	C18 (5 µm; 250x4.0 mm)	MeOH/H_2O (50:50)	1.0	(Spring et al. 2003)
15	C18 (5 µm; 250×4.6 mm)	MeOH/H_2O (70:30)	-	(Zhang et al. 2011)
		Semi-preparative separation		
16	C18 (6 µm; 300×7.8mm)	MeOH/H_2O (47:53)	1.0	(Huo et al. 2010)
17		MeOH/H_2O (52:48)		
18	C18 (250x21.2 mm)	MeCN/H_2O var.	10	(Igual et al. 2013)
19	C18 (5 µm; 250×30.0 mm)	MeOH/H_2O (70:30)	-	(Liao et al. 2012)
20	C18 (10 µm; 250x10 mm)	MeOH/H_2O var.	1.5	(Youn et al. 2014)

[a] Extended description of the chromatographic conditions in Appendix A.1; [b] structures are presented in Appendix A.2; [c] variable mobile phase conditions.

Parthenolide represents the most well studied case for the development of a synthetic aminoderivative, due to its promising biological activity which has compelled substantial efforts for its clinical application as an anticancer agent (Dell'Agli et al. 2009; Guzman et al. 2007; Guzman et al. 2005; Neelakantan et al. 2009; Woods et al. 2013).

Several authors have synthesized PTL analogs to identify a compound with improved water solubility and bioavailability. The general procedure consists in the Michael addition of an amine to the α-methylene-γ-lactone of PTL (**2**) (Figure 6).

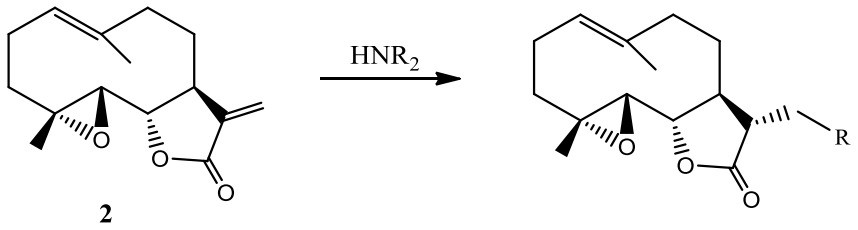

Figure 6. Synthesis of aminoparthenolide analogs.

Nasim & Crooks (2008) describe the synthesis and antileukemic properties of several parthenolide analogs derived from arylalkyl, heteroarylalkyl and alkyl amines.

Ghantous et al. (2013) demonstrated that conjugate addition of aromatics, particularly a tyramine moiety, or aliphatic amines to the α-methylene yields compounds with similar potency and better hydrophilicity than PTL. Among aliphatic acyclic derivatives, secondary amines, such as dimethylaminoparthenolide (DMAPT, **2a**) bearing a *N, N*-dimethylamino group, are more potent than are primary amines against acute myeloid leukemia (AML) cells. Furthermore, within secondary acyclic amines, analogs with at least one *N*-methyl group and short *N*-linked chains have higher anti-leukemic activity. The aliphatic acyclic amine DMAPT was considered to be the most promising analog due to increased water-solubility while retaining activity, demonstrated by the elimination of 93% of primary AML cells.

Moreover, Guzman et al. (2007) also verified the effectiveness of DMAPT in eliminating human AML stem and progenitor cells without apparent harm to normal hematopoietic stem and progenitor cells.

The abovementioned findings led to the initiation of an ongoing phase I clinical trial for DMAPT. Thus, in the present study, the semi-synthesis of dimethylaminoderivatives of compounds **6** and **7** will be performed, which will produce new data.

2.3. Biological activity: cytotoxicity

Numerous studies report on the *in vitro* cytotoxicity of STLs towards tumoral cell lines (Berger et al. 2005; Fernandes et al. 2008; Foster et al. 2011; Ghantous et al. 2013; Ghantous et al. 2010; Guzman et al. 2007; Guzman et al. 2005; Liao et al. 2012; Nasim & Crooks 2008; Rivero et al. 2003; Singh & Lai 2001; Youn et al. 2014). In some instances, STLs such as parthenolide were shown to sensitize tumour cells to radiotherapy (Mendonca et al. 2007) or to other conventional drugs (Yip-schneider et al. 2005).

However, there are only a few studies dealing with the effects of STLs on normal, non-neoplastic cells (Amorim et al. 2013). Burim et al. (1999, 2001) reported that centratherin and glaucolide B induced sister-chromatid changes and structural chromosomal aberrations *in vitro* in human peripheral blood lymphocytes, respectively. However, Guzman et al. (2005) reported that parthenolide specifically targeted leukemia cells, while sparing normal hematopoietic cells. This seems to indicate that STLs may be promising anti-neoplastic agents, with high selectivity towards tumour cells.

Furthermore, to date no information is available on the cytotoxic properties of these two guaianolides (**6** and **7**).

Bearing this in mind, it seems reasonable to begin the preliminary *in vitro* cytotoxicity studies of the present compounds on normal human fibroblasts, in order to assess their toxicity towards non-tumoral cells.

3. Materials and methods

3.1. Purification of STLs from a mixture

3.1.1. Samples preparation

The two guaianolides (**6** and **7**) were previously isolated from aerial parts of *Centaurea ornata* (Bastos 1996). The stock solutions for each compound were separately prepared by dissolving the accurately weighed reference compound to a final concentration of 10000 ppm and stored at 4^0C. This solutions were further diluted to appropriate concentrations (500 ppm) for HPLC analysis, both as separate samples and as a mixture of the two compounds, and filtered through nylon filters (diameter: 13 mm; pore dimension: 0.22 µm) from Nantong FilterBio Membrane Co., Ltd prior to injection in the HPLC column.

3.1.2. Reagents

HPLC grade acetonitrile and methanol, purchased from VWR International, were used for the HPLC analysis and the latter also for sample preparation. Before use in HPLC, distilled water was filtered through Sartolon® polyamide membrane filters (diameter: 47 mm; pore dimension: 0.45 µm) from Sartorius AG and all solvents were degassed in an ultrasonic bath at room temperature for ca. 20 min.

3.1.3. HPLC and chromatographic conditions

HPLC analyses were carried out on a Jasco modular liquid chromatograph, equipped with an intelligent HPLC pump (mod. PU-2080 plus), an autosampler (mod. AS-2057 Plus) and a diode array UV/Vis detector (mod. MD-2015, wavelength range 200 ~ 900 nm, quartz flow cell, 10 mm optical path). A VarioPrep® Nucleosil C18 (250 x 10 mm id, 5 µm particle size, 100 Å pore size) semi-preparative column from Macherey-Nagel was employed.

STLs elution was performed in isocratic and gradient mode using various gradient programs, employing the following solvent system: mobile phase A: water; mobile phase B: methanol or acetonitrile. The flow rate was 1 mL/min and the

injection volume was either 20 µL or 100µL. The column temperature was set at 25^0C. Data were processed by the software Jasco ChromPass (v. 1.8.6.1).

3.2. Semi-synthesis of aminoderivatives

3.2.1. Preparation of DMA derivatives

Treatment of ca. 10 mg of a 40 mM methanolic solution of the STL (**6** and **7**) with one equivalent of dimethylamine led to significant consumption of the STL (**6** and **7**) within 12 h at room temperature. The samples were further treated with slightly acidified (HCl) water to remove excess of dimethylamine reagent. Afterwards, the derivatives (**6a** and **7a**) were extracted with ethyl acetate and the solvent was eliminated by evaporation.

3.2.2. Nuclear Magnetic Resonance (NMR) analysis

NMR spectra of **6** and **7** were recorded on a Bruker Avance III - 400 spectrometer (operating at a frequency of 400 and 100 MHz, for ^{1}H NMR and ^{13}C NMR, respectively) while spectra of **6a** and **7a** were recorded on a Bruker ASCEND 700 spectrometer (operating at a frequency of 700 and 175 MHz, for ^{1}H NMR and ^{13}C NMR, respectively). The samples (compounds **6**, **7**, **6a** and 7**a**) were dissolved in 0.5 mL of deuterated chloroform ($CDCl_3$). Chemical shifts were reported in part per million (ppm, δ) using residual solvent signals as internal standard (7.26 ppm and 77.00 ppm, for ^{1}H NMR and ^{13}C NMR, respectively). ^{1}H-NMR, ^{13}C-NMR, DEPT-135, COSY, HSQC and HMBC correlation techniques were applied to assign the chemical shifts of proton and carbon atoms.

3.3. Cytotoxicity assays

In vitro cytotoxicity assays were performed in normal human fibroblasts (HFF-1, ATCC, SCRC1041) as a means to evaluate the potential toxicity of compound **6** towards non-neoplastic cells. In fact, evaluating the toxic side-effects of anti-neoplastic compounds, i.e. their safety, is a critical part of the drug development process. However, there is little data available concerning the toxic side-effects of

sesquiterpene lactones (Amorim et al. 2013). Parthenolide and other related compounds, that have recently reached clinical trials, are reported to show minimal non-target toxicity (Guzman et al. 2007). In the present study, the cytotoxic effect of compound **6** was tested using the resazurin assay (Riss et al. 2013), employing three drug concentrations, similar to those for which most STLs usually show therapeutic/cytotoxic activity towards neoplastic cells (Ghantous et al. 2013).

3.3.1. General cell culture conditions

In vitro assays were performed using human foreskin fibroblasts HFF-1 (ATCC, SCRC1041), grown in Dulbecco's modified Eagle's media (DMEM) (Gibco, 21885-025) supplemented with 10% (V/V) newborn calf serum (Gibco, 10270-106) and 1% (V/V) penicillin/streptomycin (biowest, L0022-100) 1% (V/V) – (DMEM+), at 37.0ºC, in a fully humidified air containing 5% CO_2. The cells were fed every 3 days. When 90% confluence was reached, cells were detached from T75 flasks using 2 mL of a 0.25% (w/V) trypsin (Sigma Aldrich, T4799-25G) - PBS 1x [EDTA (2,2',2",2'"-(Ethane-1,2-diyldinitrilo)tetraacetic acid) 0.05 % (w/V), glucose 0.1 % (w/V)] solution and resuspended in culture medium at cellular density according to the assay. All assays were performed with 6 replicates. Results are presented as mean.

Buffer PBS 1x was prepared and contains 4g NaCL, 0.1 g KCl, 0.72 g Na_2HPO_4 and 0.12 g KH_2PO_4 per 1 L dionized H_2O, with a pH of 7.4.

3.3.2. Resazurin assay

HFF-1 cells were seeded in 96 well plates (150 µL/well) at a density of 2 x 10^4 cells/mL (3000 cells/well). After reaching a state of subconfluence (24h), DMEM+ was removed and cells incubated with **6** in DMEM+ at concentrations of 0.28, 1.38 and 2.75 µM. At 24, 48, and 72h, 20 µL (1 mg/mL) resazurin (Sigma Aldrich, R7015-5G) in PBS 1x was added to each well. Cells were incubated for 3h and fluorescence ($\lambda_{ex/em}$=530/590 nm) read in a fluorometer (Biotek – Synergy Mx). Moreover, at these time-points plates were observed in an inverted fluorescence microscope (Carl Zeiss – Axiovert 200) and phase contrast images captured.

Negative control was performed incubating cells with DMEM+ and with DMEM+ containing DMSO at the maximum DMSO concentration used in the assays. The positive control was done incubating with triton 0.1 wt.%. Cell viability (%) was

calculated as follows: Fluorescence of sample/ Fluorescence of negative control * 100.

3.3.3. Statistical analysis

The cell viability (%) values for 24h, 48h and 72h, for each tested concentration, were compared using the ANOVA approach using the R-project software version 3.1.0 from Institute for Statistics and Mathematics of Wirtschaftsuniversität Wien.

4. Results and discussion

4.1. Purification of the STLs from a mixture

The chromatographic work was developed from the start using a semi-preparative column, rather than with an analytical column in the initial stages of the method development, in order to avoid time-consuming optimizations that might have been necessary when the method was scaled-up, i.e., when larger volumes were injected.

Different chromatographic conditions were investigated in order to obtain chromatograms with the best possible resolution and sensitivity within a reasonable run time, i.e., to obtain better separation of adjacent peaks within a shorter time period. On the basis of the 3D UV spectra plot, the signal intensity was higher at 210 nm, thus detection was set at this wavelength, which was also selected by other authors (Appendix A.1).

Two mobile phase systems, methanol–water and acetonitrile–water, were tested; the acetonitrile–water system did not result in a better selectivity of separation (elution) of the investigated compounds, in comparison with the methanol-water system, though it has a higher elution capacity, i.e., lower retention times are obtained. Thus, since acetonitrile is a more expensive solvent, optimizations were proceeded with methanol as the organic solvent.

Furthermore, formic or trifluoroacetic acid at low (for instance, 0.1%) concentrations are commonly added to the mobile phase to control retention of compounds. Adding acid (proton donor) increases the hydrogen ion concentration (lowers pH) of the solution, which will influence the extent of the analyte ionization. Ionised form is more polar and, as a result, less well retained under reversed phase conditions (McMaster 2007; Snyder et al. 1997). However, addition of formic acid 0.1% to the mobile phase was found to have no significant improvement on the separation.

Elution was performed isocratically (the water-methanol composition was kept constant during the separation process) as well as by using a solution gradient mode (the water-methanol composition was altered throughout the separation process, by decreasing the polarity of the mobile phase, i.e., by increasing the proportion of the organic (non-polar) solvent).

Despite the fact that isocratic conditions are most often employed in preparative HPLC to minimize operating problems, a fair number of gradient elutions have been reported for those cases in which separations are troublesome (Hostettmann et al. 1998) (Appendix A.1). In fact, for this particular separation of two hydrophobic compounds with extremely high structural and stereochemical similarity, the most satisfactory results were obtained using a gradient elution. Different gradient mode compositions, using H_2O (A)/ MeOH or MeCN (B) as the mobile phase, employed for the HPLC analysis of STLs **6** and **7** are presented in Appendix A.3.

Moreover, four sets of chromatograms using two different gradient-elution compositions, illustrated in Figure 7, are shown in Figure 8.

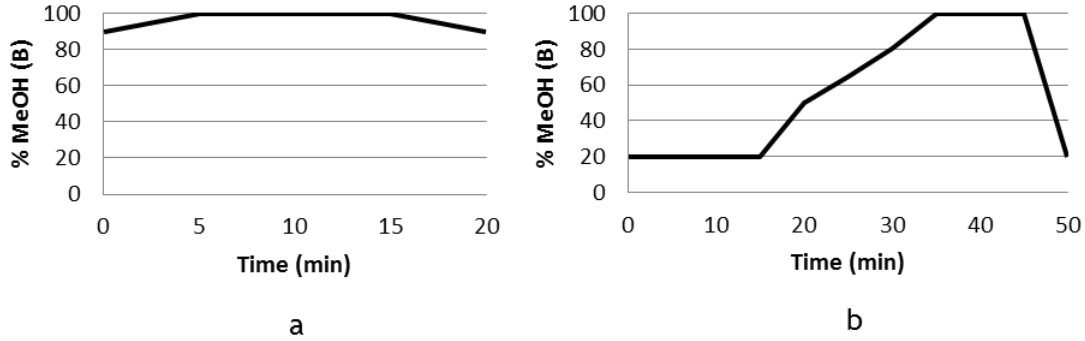

Figure 7. Gradient elution programs employed to obtain the chromatographic separations. (a) Gradient program 1; (b) gradient program 2.

In Figure 8 (a, b) the two STLs, **6** and **7**, were analyzed independently, while in Figure 8 (c, d) these compounds where in a mixture 1:1, thus a single sample was analyzed. With the mobile phase variation applied in Figure 8a, a R_T of 13.21 min for **6** and 13.27 min for **7** was obtained, from which can be concluded that the two molecules would co-elute if present in the same sample. The testing of different chromatographic conditions led to the observation that shortening the run time compromised the separation of the two compounds. It was also verified that both compounds elute when the percentage of methanol in the mobile phase is 100%.

Comprehensive optimizations lead to the separation obtained in Figure 8b, where a R_T of 41.05 min for **6** and 42.09 min for **7** was obtained. Even though the difference in the retention times of **6** and **7** was not considerable, with peaks with relatively low resolution, and the run time was not as short as ambitioned at first, purification studies aiming at the isolation of a pure substance from the mixture

were continued. Thus, the separation obtained in Figure 8c, where a R_T of 40.81 min for **6** and 41.82 min for **7** was achieved, confirmed that under the same chromatographic conditions, separation of the substances from a single sample did not undergo substantial modifications.

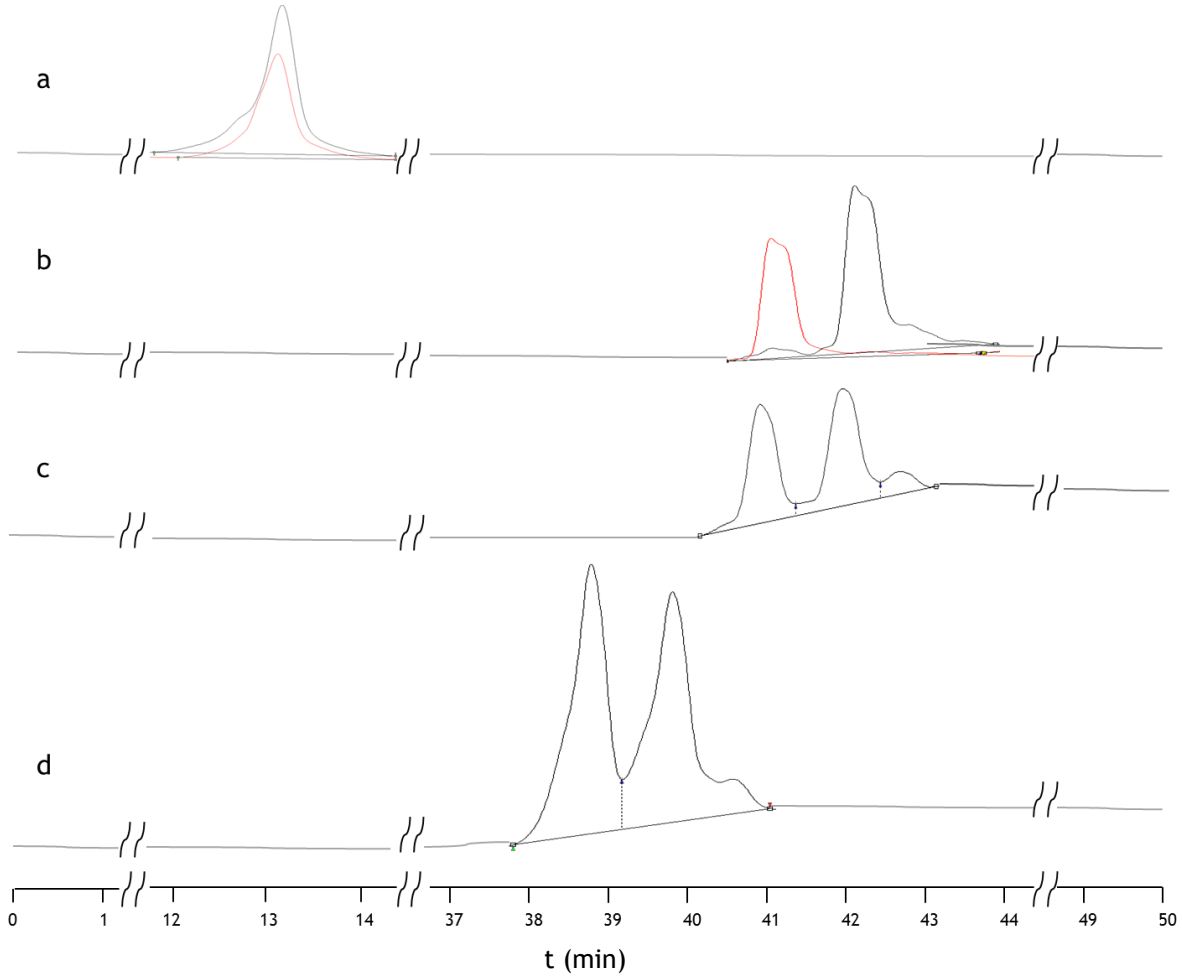

Figure 8. HPLC chromatograms obtained for the separation of 6 and 7. Conditions: A: gradient elution (Figure 7a), 20 µL injection, separate samples; B: gradient elution (Figure 7b), 20 µL injection, separate samples; C: gradient elution (Figure 7b), 20 µL injection, **6** and **7** in mixture (1:1); D: gradient elution (Figure 7b), 100 µL injection, **6** and **7** in mixture (1:1).

Within the context of this work, the main advantage of using semi-preparative chromatography is the possibility of having high levels of sample load, which would ultimately result in the recovery of pure substances. For such, the large volumes of eluted solvent would require a suitable fraction collector for its handling.

In this sense, a 100 µL sample of the mixture of the two compounds was loaded onto the semi-preparative column and the separation in Figure 8d was

attained. A decrease in chromatographic resolution due to peak width broadening is observed leading to a more significant co-elution of **6** and **7**, which translates into inability to separate these two compounds using a RP_{18} column. On the other hand, a reduction in the retention times of **6** (38.81 min) and **7** (39.89 min) was observed. Minor variations in the retention times of the compounds (ca. 1 min) were expected since the samples were analyzed on different days and the column pressure may vary. Additionally, throughout the period of the present work, solvents from different batches are used and the characteristics of the distilled water may vary. Nonetheless, in the separation showed in Figure 8d there was a decrease of approximately 2 min in the R_T of both STLs when compared to the separation presented in Figure 8c. This may be explained considering that a larger quantity of sample (more mass of compound) was loaded onto the column impairing the interaction of some molecules with the stationary phase, which eluted earlier, thus carrying the rest of the sample, and ultimately resulting in lower retention times.

Fardella et al. (1999) also analyzed two guaianolides - grosheimin and deacylnaropicrin (compounds **8** and **9**; Appendix A.1 and A.2) - structurally similar between them and similar to **6** and **7** and likewise it was verified that their retention times presented only a small difference (0.67 min).

These results may be explained considering the mechanism by which the octadecyl (C18) carbon chain – bonded silica stationary phase interacts with the molecules in the mobile phase. The major interactions that take place between the two phases are hydrophobic interactions, which consist in induced dipole-dipole forces between the aliphatic chains of the modified silica particles and the analyte molecules. Consequently, these interactions do not distinguish between a compound with a hydroxyl group and one with a carbonyl group in its turn. Given the fact that this is the only chromatographically detectable difference between these STLs, the used C18 column does not possess selectivity that allows for their effective separation.

In light of the aforementioned findings, an octyl (C8) column which exhibits lower hydrophobicity when compared to the C18 column (lower intensity of hydrophobic interactions and shorter retention times) may also not be suitable for the separation of these two guaianolides under the same mobile phase conditions.

In a new approach to this problem, a column that allows for multiple interaction mechanisms between the coating particles and the molecules, such as a biphenyl bonded silica stationary phase column, should be considered.

The biphenyl phase is composed of two phenyl groups bonded end-to-end and an aryl linker to the silica particles. Its main advantage is the ability to resolve compounds using π–π interactions, commonly referred to as aromatic selectivity, resulting in different selectivity to alkyl phases like C18. When compared to single phenyl ring phases, it is more hydrophobic and provides a larger electron cloud, therefore maximizing hydrophobic retention and increasing the area available for π–π interactions, providing the highest degree of aromatic selectivity available (Chust 2014; Restek Corporation 2008). When applied to this particular separation, the π electrons of the phenyl group would likely interact with the π electrons in the double bond (C=O) present in compound **6**, favoring its retention and, thus, increasing the difference between the retention times of **6** and **7**, making it possible for their semi-preparative separation.

Furthermore, in HPLC, the mobile phase can be altered to enhance a separation or to obtain a desired resolution. With the biphenyl phase, this can be achieved with simple mobile phase changes. The choice of organic solvent used in the mobile phase can alter the selectivity by switching between two separation mechanisms: dispersive (hydrophobic) interactions and π–π interactions. Whereas using acetonitrile makes a biphenyl column more C18-like in its retention and selectivity, due to the triple bond (C≡N) which restrains π–π interactions of the analyte with the stationary phase, methanol prompts aromatic selectivity (π–π interactions) (Chust 2014; Restek Corporation 2008).

Thus, instead of attempting to expand the use of the traditional C18 column with numerous additives and running conditions alterations that is time-consuming, this separation can be further transformed with the selectivity and performance that the biphenyl column would provide.

4.2. Semi-synthesis of aminoderivatives

The semi-synthesis of the dimethylaminoderivatives of STLs **6** and **7** was performed so that, afterwards, it is possible to compare the bioactivity of these compounds prior to and after derivatization.

N, N-dimethylaminogrosshemin-2,3-dihydroxyisobutyrate (**6a**) and *N, N*-dimethylaminodehydrogrosshemin-2,3-dihydroxyisobutyrate (**7a**) were obtained through a Michael addition of dimethylamine to the α-methylene-γ-lactone ring,

more specifically to the electrophilic vinylic carbon C-13, of **6** and **7**, respectively (Figure 9).

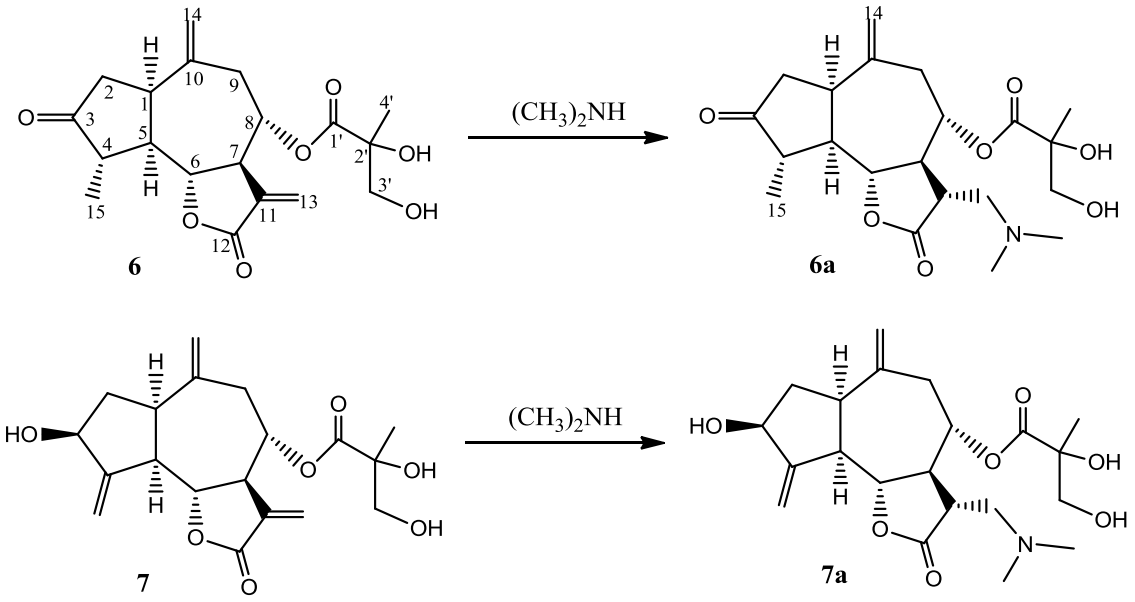

Figure 9. Semi-synthesis of the diamino derivatives of STL 6 (6a) and STL 7 (7a).

Structure elucidation has an important role in all of the studies concerning the full characterization of STLs, namely in the confirmation of semi-synthesis of derivatives. Thus, the complete characterization of STLs **6** and **7** by NMR was previously performed, including different experiments: ^{1}H-NMR, ^{13}C-NMR, HSQC, COSY and HMBC in order to verify the anticipated modifications in the NMR spectra of the respective derivatives, **6a** and **7a**, respectively.

Sesquiterpene lactones **6** and **7** have a C_{15} guaianolide backbone, with a 2,3-dihydroxyisobutyrate side-chain at C-8, presenting only slight structural differences in the cyclopentene ring (C-1 to C-5), which, consequently, yields very similar ^{1}H-NMR and ^{13}C-NMR records (Figure 10 and 11). This similarity is evidenced in the ^{13}C-NMR spectra presented in Figure 10 (b, c). Major differences are observed at the C-3, C-4 and C-15 level. While compound **6** exhibits a ketone carbonyl carbon (C-3 at δ 218.32 ppm), a methynic carbon (C-4 at δ 46.97 ppm) and a methylic carbon (C-15 at δ 15.51 ppm), in compound **7** the unsaturation occurs at the C-4 and C-15 (quaternary carbon and methylenic carbon, respectively at δ 152.16 and 114.24 ppm) level and C-3 is a methynic carbon (CH) with a chemical shift (δ 73.75 ppm) typical of a carbon directly bonded to an oxygen atom.

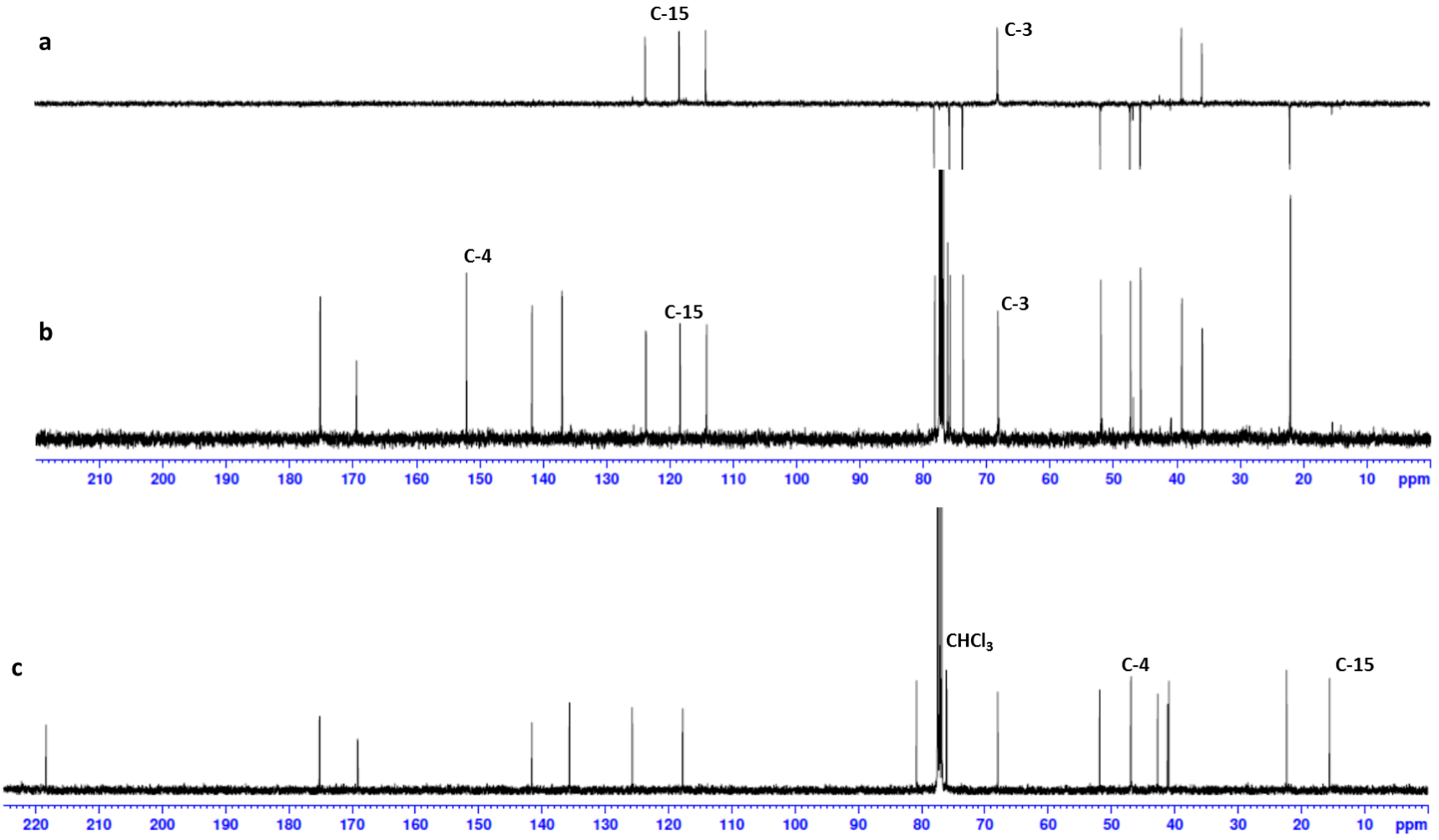

Figure 10. ^{13}C NMR spectra ($CDCl_3$) of STLs 6 and 7. (a) DEPT-135 sprectrum of STL **7**; (b) normal ^{13}C NMR spectrum of STL **7**; (c) ^{13}C NMR spectrum of STL **6**.

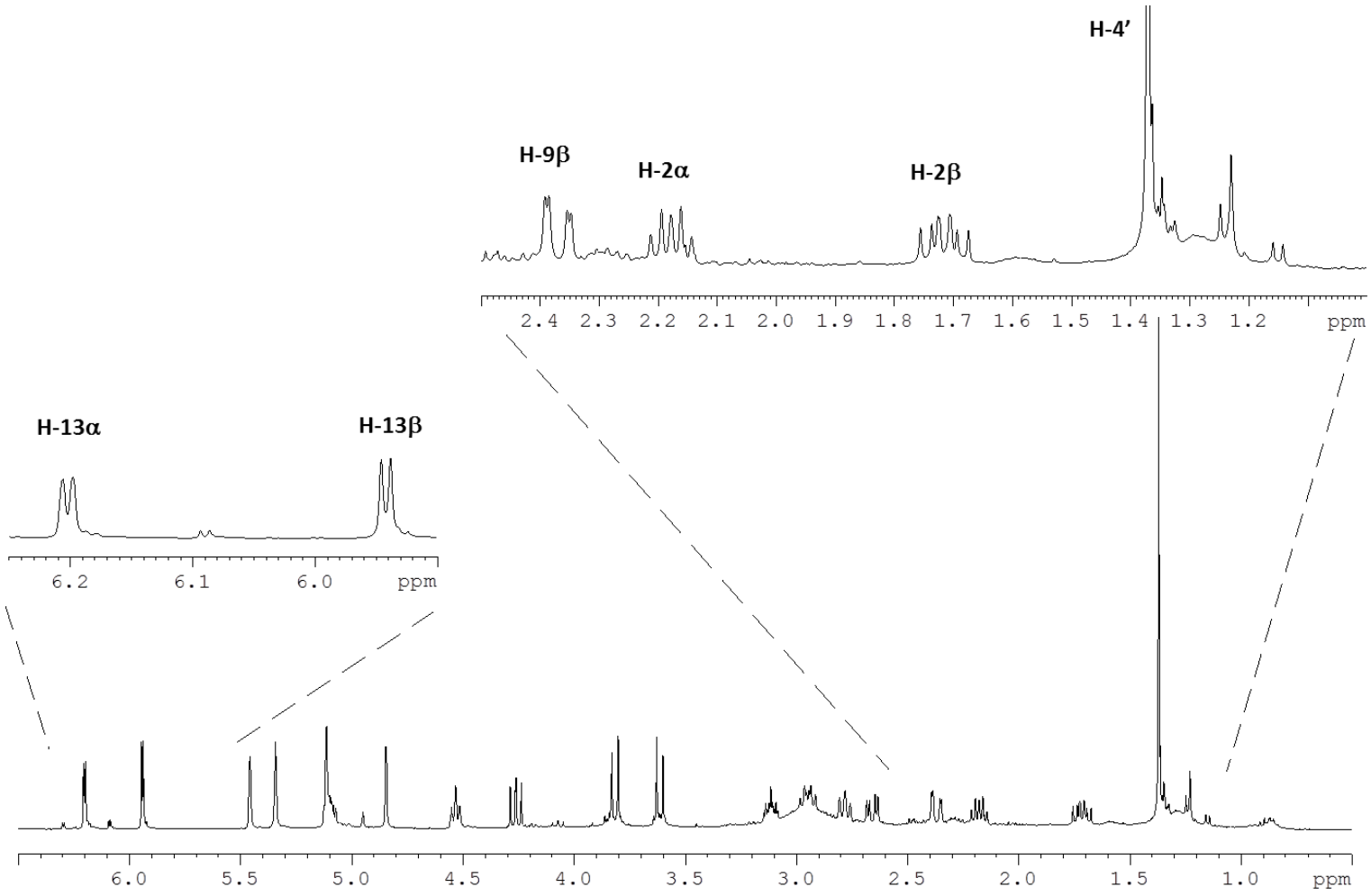

Figure 11. ^{1}H NMR spectrum ($CDCl_3$) of STL 7 with amplifications of two regions.

Figure 10a shows the DEPT-135 spectrum of compound **7** that allows for the distinction between the four types of carbons: methynic (CH) and methylic (CH_3) as positive signals; methylenic (CH_2) as negative signals; and eliminating quaternary signals (for instance C-4), which are identified by comparison with the ^{13}C-NMR spectrum.

Likewise, the ^{1}H-NMR spectra of both compounds present an array of similar signals, namely the ones corresponding to the α-methylene-γ-lactone ring: two olefinic protons corresponding to H-13ß an H-13α, in the region between δ 5.6–6.5 ppm.

The ^{1}H-NMR spectrum of compound **7**, with amplifications of the regions between δ 5.9-6.2 and 1.0–2.5 ppm, is presented in Figure 11. These two regions undergo significant modifications upon preparation of the *N, N*-dimethylamino derivatives.

Hence, the detailed analysis of the ^{1}H NMR spectra of **7a**, showed the absence of the two olefinic proton resonances of **7** at δ 5.95 and 6.20 ppm (corresponding to H-13ß an H-13α, respectively) and the appearance of one methynic and two non-equivalent methylenic protons at δ 2.33-2.37, 2.09 and 2.06 ppm (corresponding to H-11, H-13α an H-13 ß, respectively). Another evidence is the appearance of a singlet at δ 2.66 ppm due to the resonance of the two *N*-methyl groups. Furthermore, in the ^{13}C NMR spectrum, besides the appearance of a signal due to the two magnetic equivalent *N*-methyl carbons at δ 34.84 ppm, the shift of carbon C-11 resonance from δ 137.04 ppm to δ 45.48 ppm (a 92.56 ppm difference) and carbon C-13 resonance from δ 123.81 ppm to δ 57.77 ppm (a 66.04 ppm difference) can be highlight.

The ^{13}C NMR and ^{1}H NMR assignments (δ in ppm) around the α-methilene γ-lactone, for compounds **6** and **7**, and their dimethylaminoderivatives **6a** and **7a**, are represented in Figure 12. Accordingly, these data confirm that the amination of the conjugated exocyclic double bond of **6** and **7** had occurred.

Elucidative amplifications of HSQC, COSY and HMBC spectra of compound **7** can be found in Appendix A.4 while the complete attribution of the ^{13}C NMR and ^{1}H NMR data of the four compounds (**6, 7, 6a** and **7a**) are present in Appendix A.5.

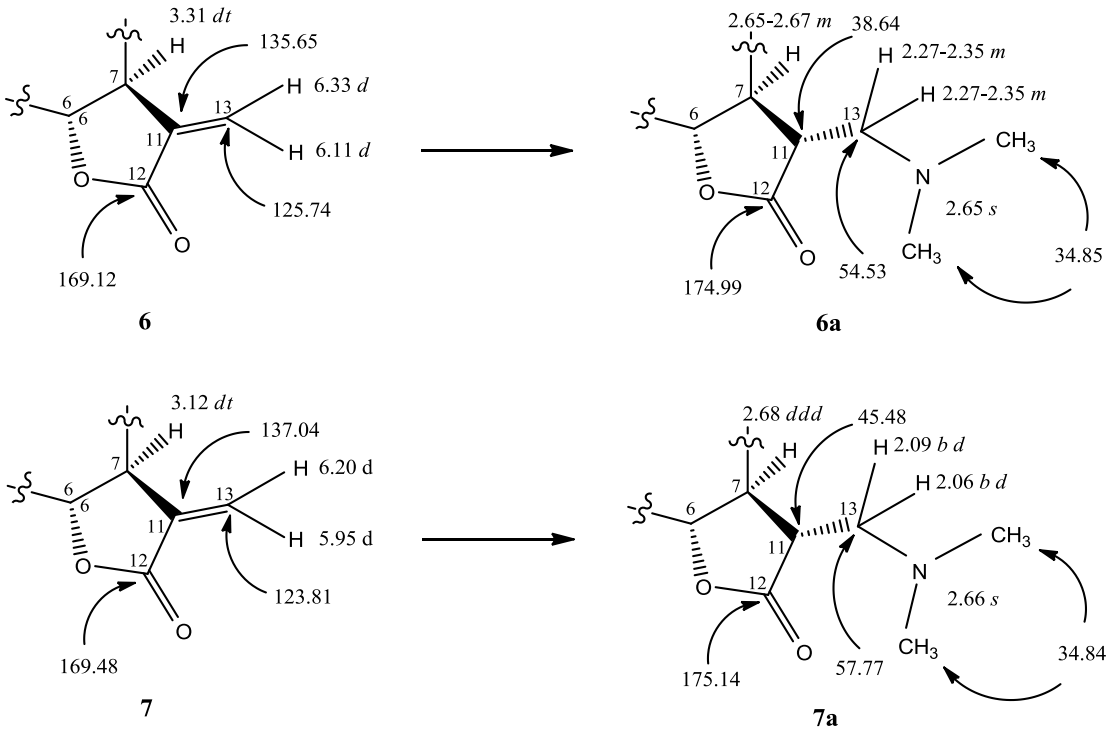

Figure 12. ^{1}H and ^{13}C NMR assignments (δ in ppm) for compounds 6, 7 and their diamino derivatives (6a and 7a).

4.3. Cytotoxicity assays

In spite of the ever-increasing evidence that STLs have anti-tumoral properties, the extensive reactivity of these compounds also results in toxic effects towards humans, which must be carefully assessed to ensure their safe utilization (Amorim et al. 2013).

The structure-activity relationship of STLs for the cytotoxic and anti-tumoral effects is well studied, being attributed to the α-methylene-γ-lactone group. Moreover, the lipophilicity of these substances can considerably influence pharmacokinetic studies.

In the present study, i*n vitro* cytotoxicity assays were performed in normal human fibroblasts as a means to evaluate the potential toxicity of compound **6** towards non-tumoral cells.

Observation of cultured cells on an inverted fluorescence microscope (Carl Zeiss – Axiovert 200) did not reveal any conspicuous differences between control and exposed cells at any time-point. Phase contrast images captured by observation of the plates are presented in Figure 13. No significant alterations in the morphology of

the cells are observed when comparing cells exposed to 2.75 μM with control cells, at 24h (**a** and **b**, respectively), 48h (**c** and **d**, respectively) and 72h (**e** and **f**, respectively).

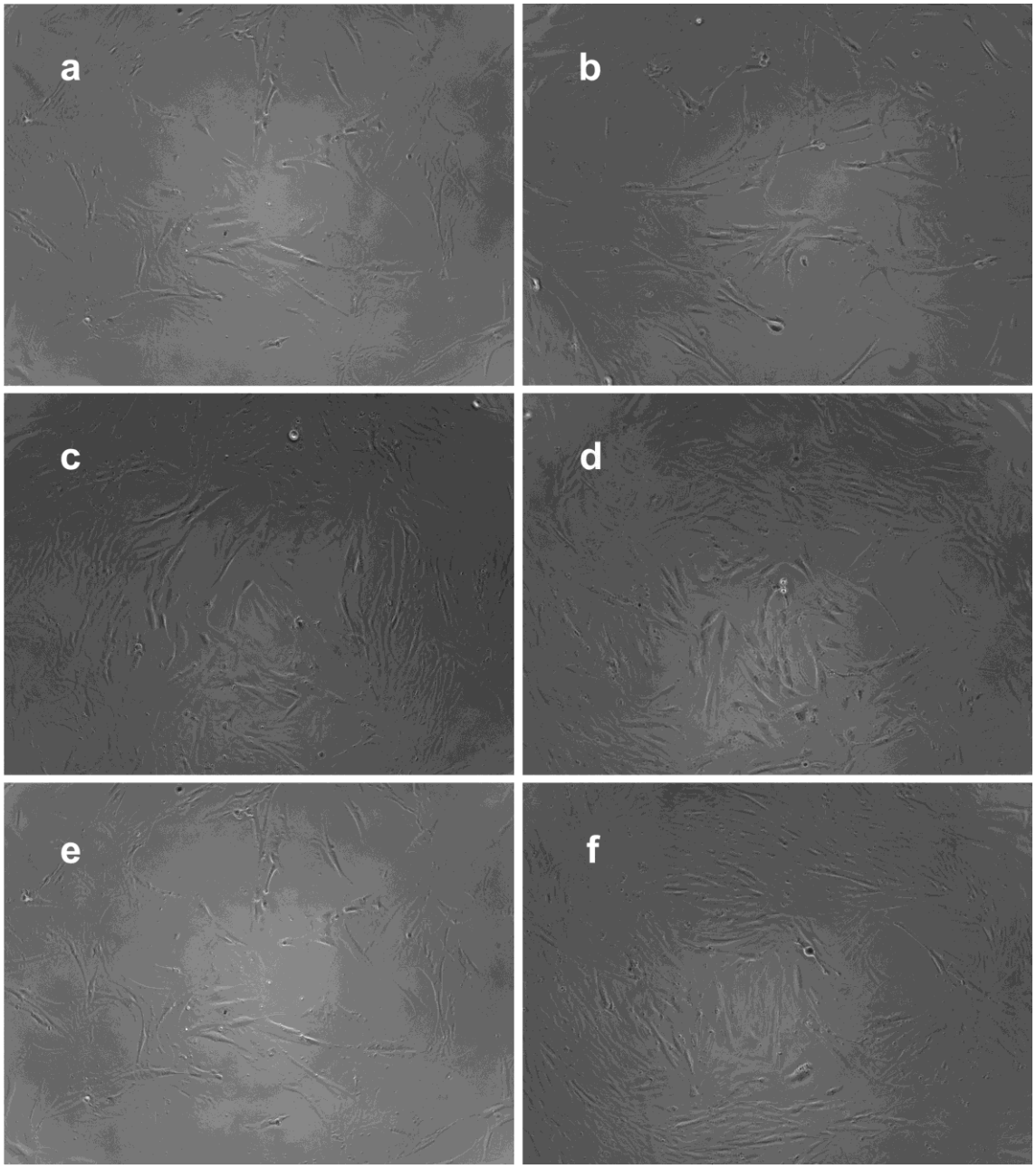

Figure 13. *In vitro* cytotoxicity experiments on human foreskin fibroblasts. (a), (c) and (e) Control cells (DMEM+/DMSO) at 24h, 48h and 72h, respectively; (b), (d) and (f) cells exposed to 2.75 μM of compound **6** at 24h, 48h and 72h, respectively. Inverted fluorescence microscopy 400x.

The percent viability of cells, for time-points at 24, 48 and 72 hours, treated with three different concentrations of compound **6** (0.28, 1.38 and 2.75 μM) is plotted in Figure 14.

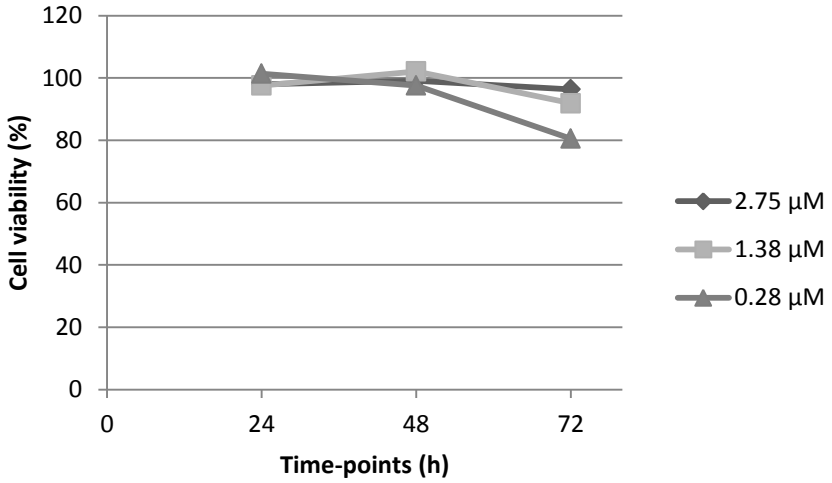

Figure 14. *In vitro* cytotoxicity experiments on human foreskin fibroblasts. The viability of cells exposed at 24h, 48h and 72h, to 0.28, 1.38 and 2.75 μM of compound **6** is expressed as a percentage of control cells (DMEM+/DMSO) viability.

The statistical analysis revealed that for the higher concentration of **6**, 2.75 μM, that the cells were exposed to, as well as for 1.38 μM, there was no significant differences between the percentage of viable cells at 24, 48 and 72 hours ($p>0.05$). On the other hand, contrary to expected, in the lower concentration of **6**, 0.28 μM, that the cells were exposed to there was significant differences between the viability of cells at 24, 48 and 72 hours ($p<0.05$). Thus, it is assumed that an error occurred, considering that the lower the concentration, the more errors are associated with the measurement.

The phase contrast images have a morphological visual aspect and, hence, are illustrative of the results demonstrated in Figure 14. Moreover, they complement the cell viability (%) results.

These preliminary data suggest that this sesquiterpene lactone is not toxic towards normal human foreskin fibroblast cells in concentrations of up to 2.75 μM after a period of incubation from 24h – 72h, i.e., it does not show off-target toxicity. Moreover, these results are in accordance with the ones reported by other authors

concerning parthenolide and other related compounds that have recently reached clinical trials, which presented minimal non-target toxicity (Guzman et al. 2007).

Nevertheless, further experiments should be performed, including treatment of cells with higher concentrations of compound **6**, the use of a dispersant since the compound is lipophilic and eventually extend the time-points at which the fluorescence of the plates is read.

5. Conclusions

5.1. General conclusions

The main interest in sesquiterpene lactones is related to their anti-tumoral potential, since some of the STLs have been found to show enough potential to enter clinical trials. However, they present low oral bioavailability, due to poor water-solubility, which translates into the need to synthesize more water-soluble derivatives of these compounds, more specifically aminoderivatives. On the other hand, the extensive reactivity of STLs also results in toxic effects towards humans, which require careful assessment to ensure their safe utilization.

Semi-preparative separation of the two guaianolides (**6** and **7**) was attempted and different mobile phase conditions were tested. To address the purpose of the semi-preparative chromatography, a 100 µL sample of the mixture of the two STLs was loaded onto the C18 semi-preparative column. A decrease in chromatographic resolution due to peak width broadening was observed leading to a more significant co-elution of **6** and **7**, with retention times of 38.81 min and 39.89 min, respectively, which translates into inability to separate these two compounds using a C18 column under the tested chromatographic conditions.

Furthermore, *N, N*-dimethylaminogrosshemin-2,3-dihydroxyisobutyrate (**6a**) and *N, N*-dimethyl-aminodehydrogrosshemin-2,3-dihydroxyisobutyrate (**7a**) were obtained through a Michael addition of dimethylamine to the α-methylene-γ-lactone ring, more specifically to the electrophilic vinylic carbon C-13, of **6** and **7**, respectively.

The amination of the conjugated exocyclic double bond of **6** and **7** was confirmed by comparison of their ^{13}C NMR and ^{1}H NMR spectra with the complete NMR characterization of STLs **6** and **7**. This analysis showed, for instance, the appearance of a singlet at δ 2.66 ppm due to the resonance of the two *N*-methyl groups, in the ^{1}H NMR spectra, whereas in the ^{13}C NMR spectra the appearance of a signal due to the two magnetic equivalent *N*-methyl carbons at δ 34.8 ppm can be highlighted.

Finally, the preliminary *in vitro* cytotoxicity studies of compound **6** were performed on normal human fibroblasts, in order to assess their toxicity towards non-tumoral cells. At the maximum concentration (2.75 µM) that the cells were exposed to, phase contrast images captured by observation of the plates on an inverted

fluorescence microscope revealed no significant alterations in the morphology of the cells when comparing cells exposed to 2.75 μM with control cells. In addition to the percent viability of cells data, these preliminary results indicate that STL **6** does not possess toxicity towards normal human foreskin fibroblast cells in concentrations of up to 2.75 μM after a period of incubation from 24h to 72h.

5.2. Limitations and future work

Throughout the present work some setbacks were found, regarding mainly the HPLC equipment, which were fortunately solved in due time.

In a new approach to the separation of STLs **6** and **7** by semi-preparative HPLC, instead of attempting to expand the use of the traditional C18 column with numerous additives and running conditions alterations that is time-consuming, this separation may be further transformed with the selectivity and performance of a biphenyl column.

If the separation is achieved, for the recovery of pure substances, the large volumes of eluted solvent would require a suitable fraction collector for its handling, which has already been set to operate, and an efficient evaporation method for multiple samples, such as a Multivapor that is available at LEPABE.

Concerning the cytotoxicity assays, further experiments should be performed, including treatment of cells with higher concentrations of compound **6**, the use of a dispersant since the compound is lipophilic and eventually extend the time-points at which the fluorescence of the plates is read. Likewise, cytotoxicity evaluation of compound **7** and of the dimethylaminoderivatives, **6a** and **7a**, should be executed.

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Appendix

A.1. Chromatographic conditions employed by several authors for the analysis of STLs using HPLC

Table 3. Extended description of the chromatographic conditions employed by several authors for the analysis of STLs using HPLC.

Column	Mobile phase	Gradient mode	Flow rate (ml/min)	Injection volume (µL)	HPLC equipment	λ (nm)	T (^{0}C)	Analyte structure	Retention time (min)	Reference
				Analytical separation						
C18 (10 µm; 200x4.0 mm)	MeOH/H_2O (30:70)	-	0.8	10	410 LC quaternary pump (Perkin Elmer); LCJ 90 UV detector (Perkin Elmer)	230	-	8,9	1: 7.3 2: 6.7	(Fardella et al. 1999)
C18 (5 µm; 250x3.0 mm)	A: MeOH/H_2O 14/86 (v/v) B: MeOH/H_2O 64/36 (v/v)	t = 0 - 20 min, 100–58% A; t = 20 - 30 min, 58% A; t = 30 - 45 min, 58–0% A; t = 45 - 50 min, 0% A; t = 50 - 52 min, 0–100% A; t = 52 - 62 min, 100% A (postrun)	0.5	20	Jasco; 2 binary pumps (mod.PU-1580); Autosampler (mod. AS-2055 Plus); DAD detector (mod. MD-1510, quartz flow cell, 10 mm optical path)	260	25	10	17.7	(Ferioli & D'Antuono 2012)
C18 (5 µm; 250x4.6 mm)	A: IPA/MeCN/ MeOH/ H_2O, 0.3% HCOOH 18:30:12:40 (v/v) B: H_2O, 0.3% HCOOH	t = 0 – 35min, 8-48% A	1.0	20	HP 1100 HPLC system; Quaternary pump G1311A; UV/DAD detector G 1315A	254	25	11	37.0	(Fritsche et al. 2002)

C18 (5µm; 250×4.6 mm)	MeCN/H_2O (55:45)	-	1.0	10	Agilent 1200 system; Quaternary solvent delivery system; Auto-sampler; DAD detector	210	40	12	ca. 16.5	(Huo et al. 2010)
C18 (5 µm; 250x4.6 mm)	A: H_2O, 0.1% H_3PO_4 B: 90% MeCN, 10% H_2O	t = 0 – 60 min, 99:1 (A:B) - 48:52 (A:B)	1.0	15	DAD detector	200	35	13	15.8	(Sessa et al. 2000)
C18 (5 µm; 250x4.0 mm)	MeOH/H_2O (50:50)	-	1.0	-	Sykam S-1000; Shimadzu SPD-M10A DAD detector	225/ 265	-	14	13.6	(Spring et al. 2003)
C18 (5 µm; 250×4.6 mm)	MeOH/H_2O (70:30)	-	-	20	Beckman GOLD series; Dual solvent pump (model 125 solvent module); DAD detector (mod. 168)	225	T room	15	9.5	(Zhang et al. 2011)
Semi-preparative separation										
C18 (6 µm; 300×7.8mm)	MeOH/H_2O (47:53)	-	1.0	10	Agilent 1200 system; Quaternary solvent delivery system; Auto-sampler; DAD detector	210	40	16	ca. 20.5	(Huo et al. 2010)
	MeOH/H_2O (52:48)	-						17	ca. 21.0	
C18 (250 x21.2 mm)	A: H_2O B: MeCN	t = 0–44 min, 10–100% B	10	1000	Shimadzu SLC-10 Avp; SPD 10 Avp DAD detector	-	-	18	30.0	(Igual et al. 2013)
C18 (5 µm; 250×30.0 mm)	MeOH/H_2O (70:30)	-	-	-	Agilent-1100 MWD; Shimadzu UV - 2401PC detector	-	-	19	-	(Liao et al. 2012)
C18 (10 µm; 250x10 mm)	MeOH/H_2O	-	1.5	-	Beckman Coulter Gold-168 system; DAD detector	-	-	20	96.0	(Youn et al. 2014)

A.2. Structures of sesquiterpene lactones analyzed by HPLC by several authors

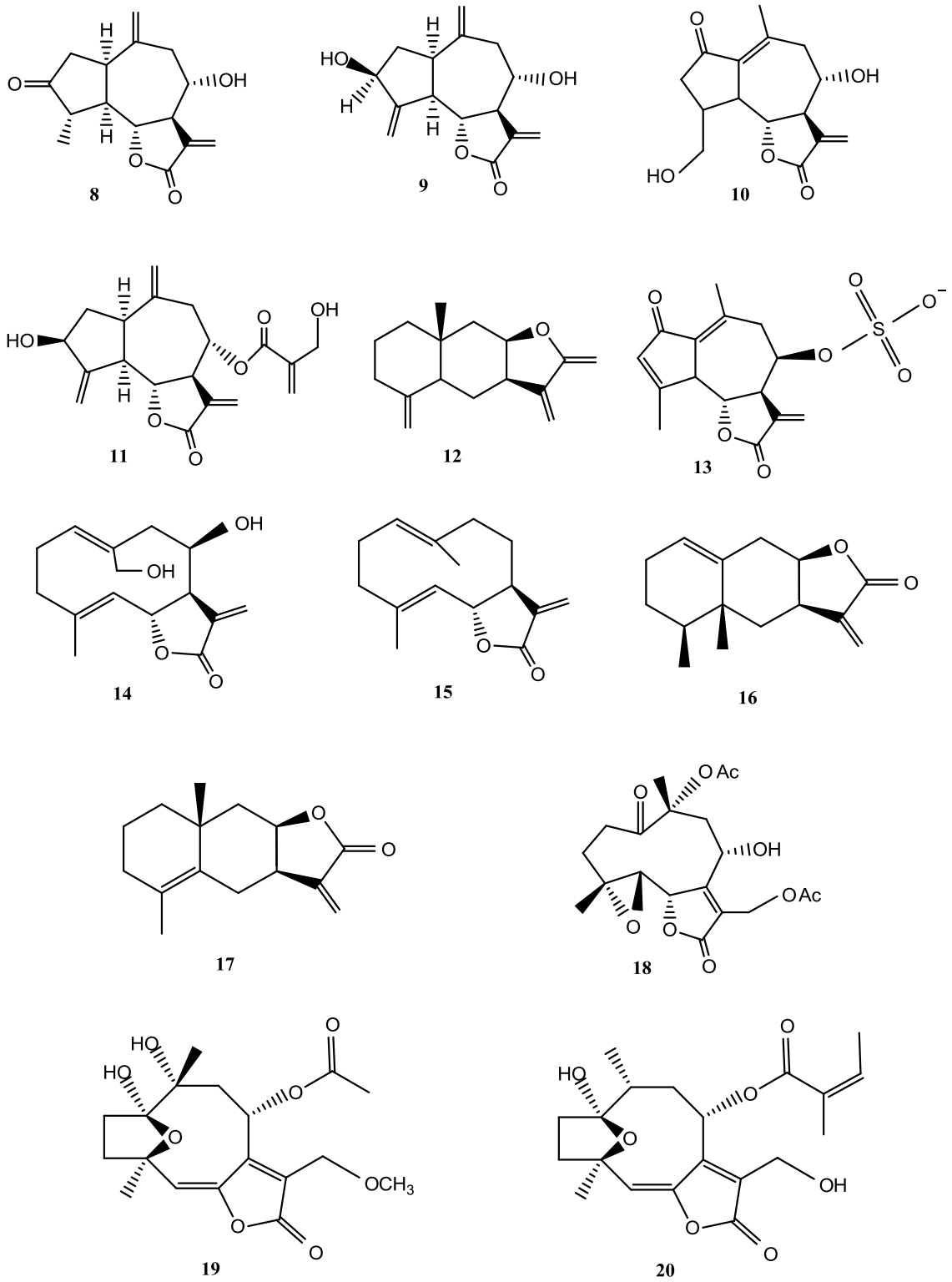

Figure 15. Chemical structures of sesquiterpene lactones analyzed by HPLC under the conditions of Table 2.

A.3. Gradient mode programs for the chromatographic separation of compounds 6 and 7.

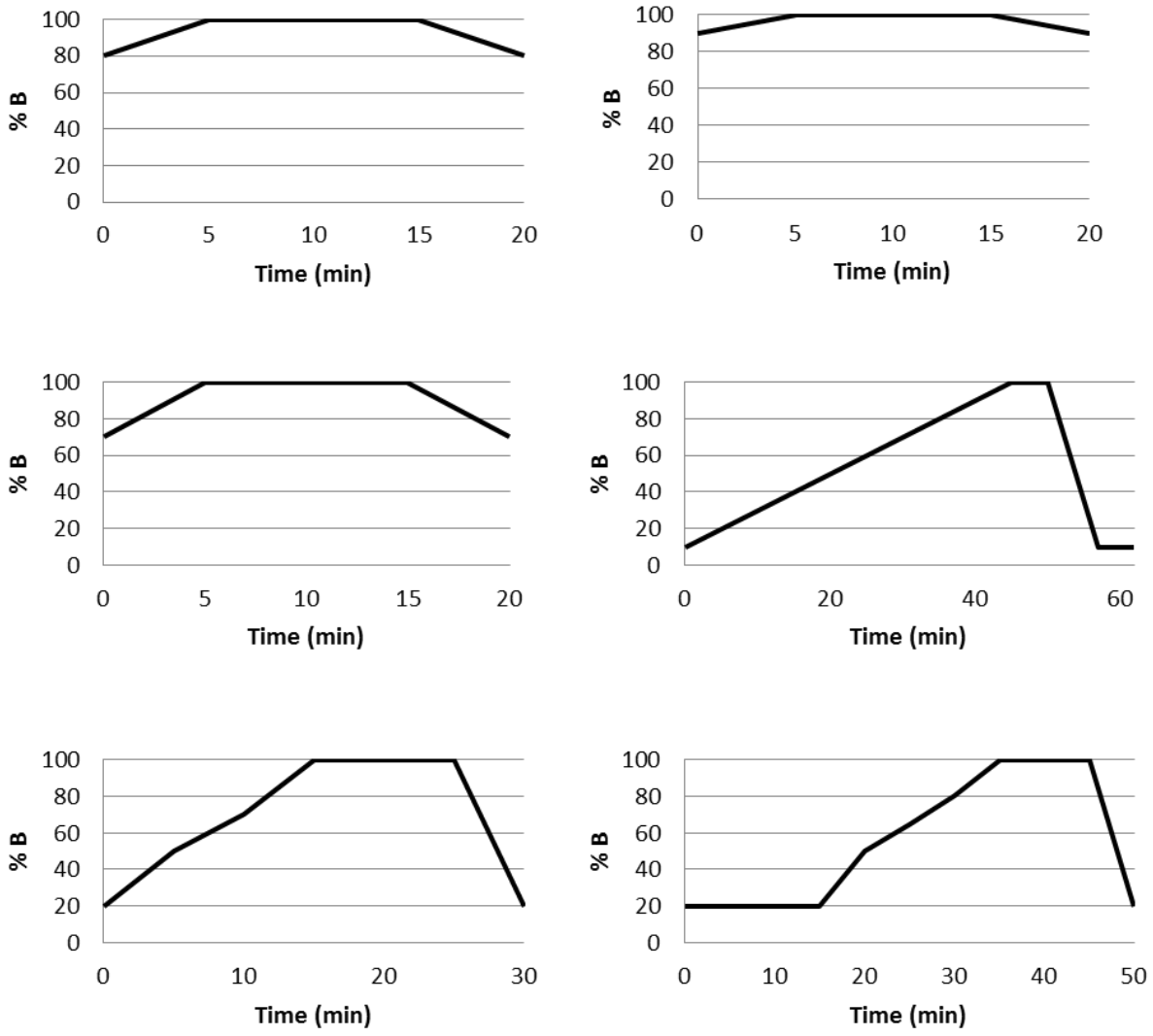

Figure 16. Different gradient mode compositions, using H_2O (A)/ MeOH or MeCN (B) as the mobile phase, employed for the HPLC analysis of STLs 6 and 7.

A.4. Amplifications of HSQC, COSY and HMBC spectra of compound 7

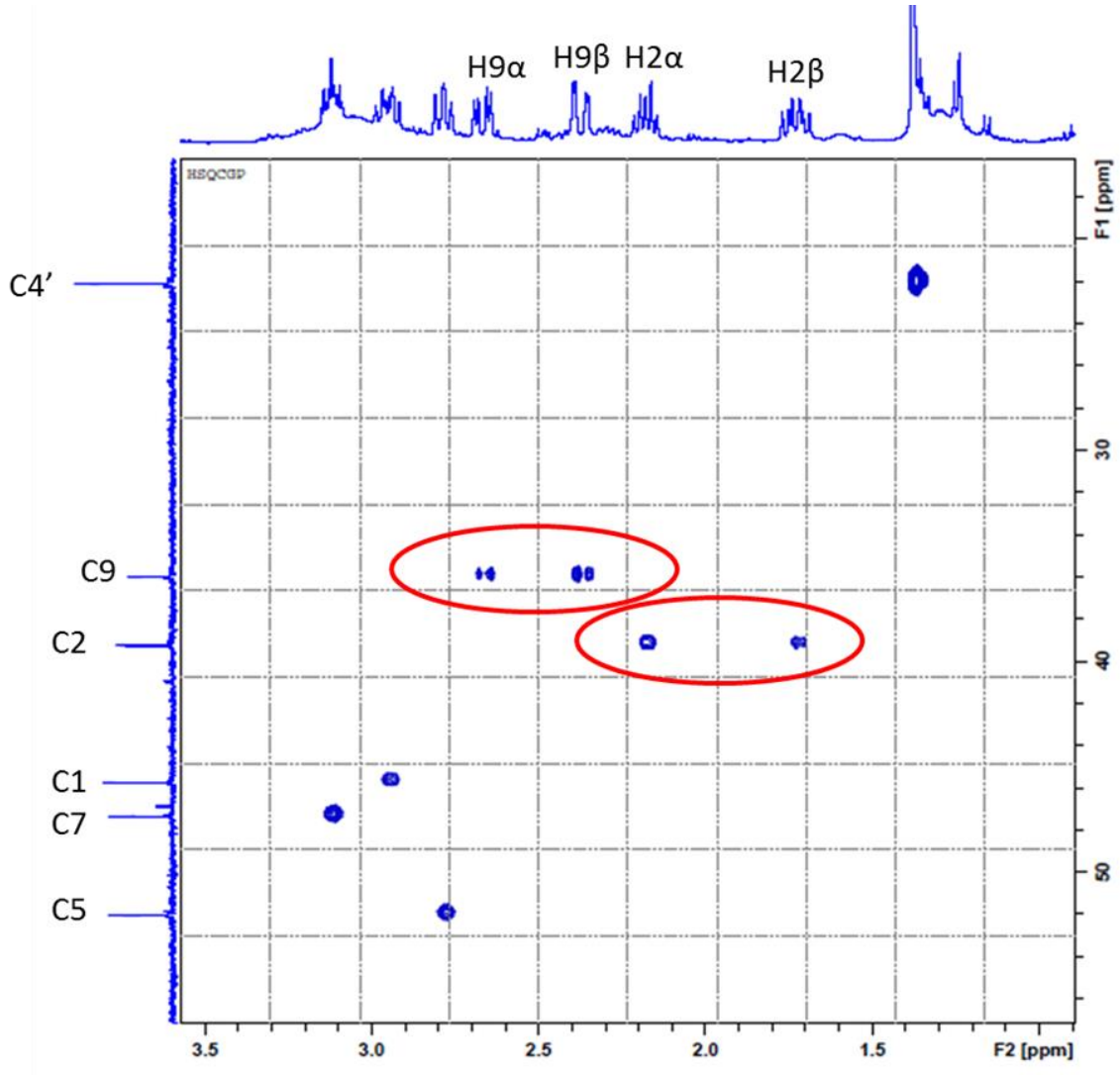

Figure 17. 2D NMR–HSQC spectrum ($CDCl_3$), for compound 7, with amplifications between 1-3.5 ppm (^{1}H) and 18-56 ppm (^{13}C).

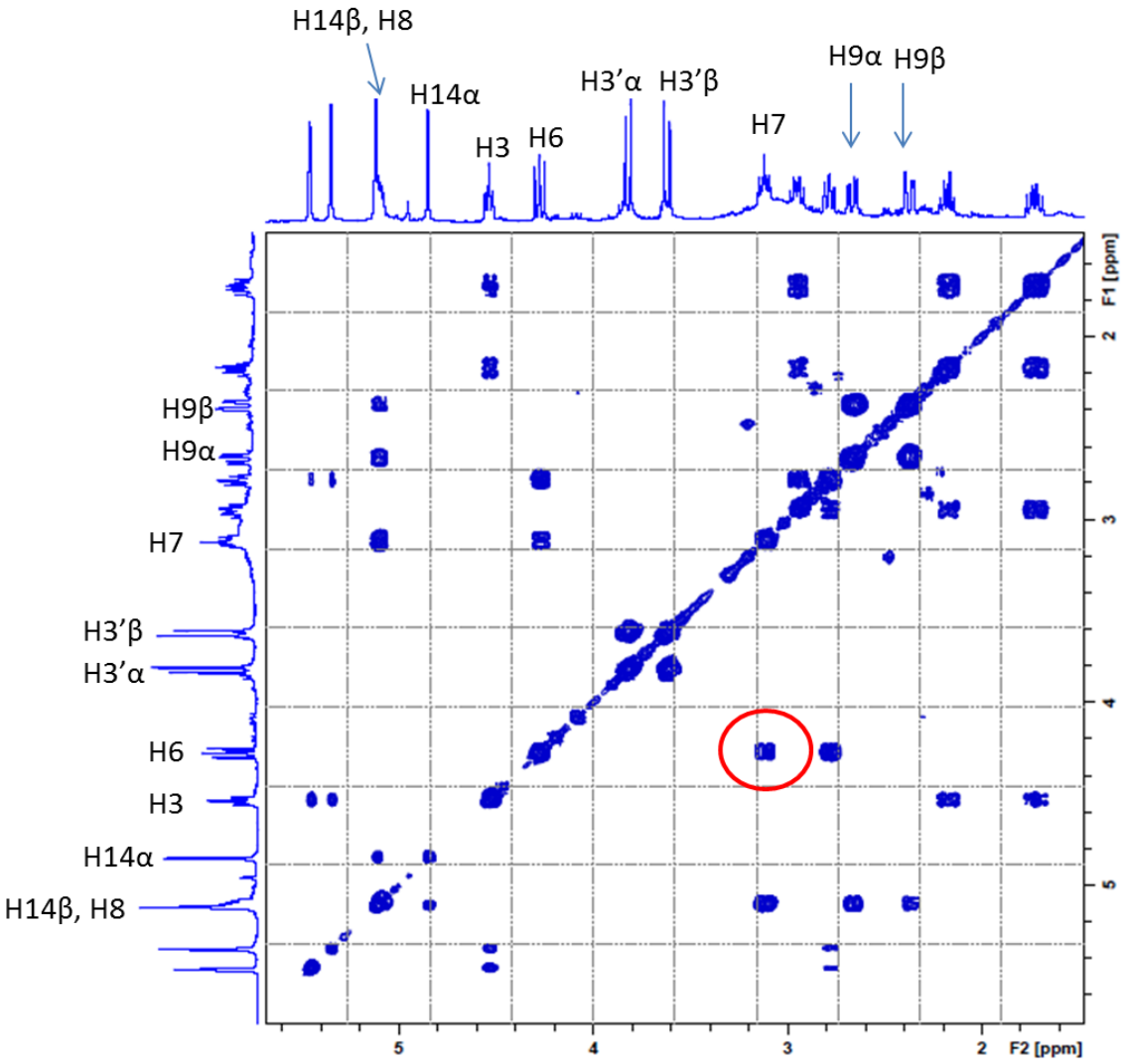

Figure 18. 2D NMR–COSY spectrum ($CDCl_3$), for compound 7, with amplifications between 1-6 ppm (^1H).

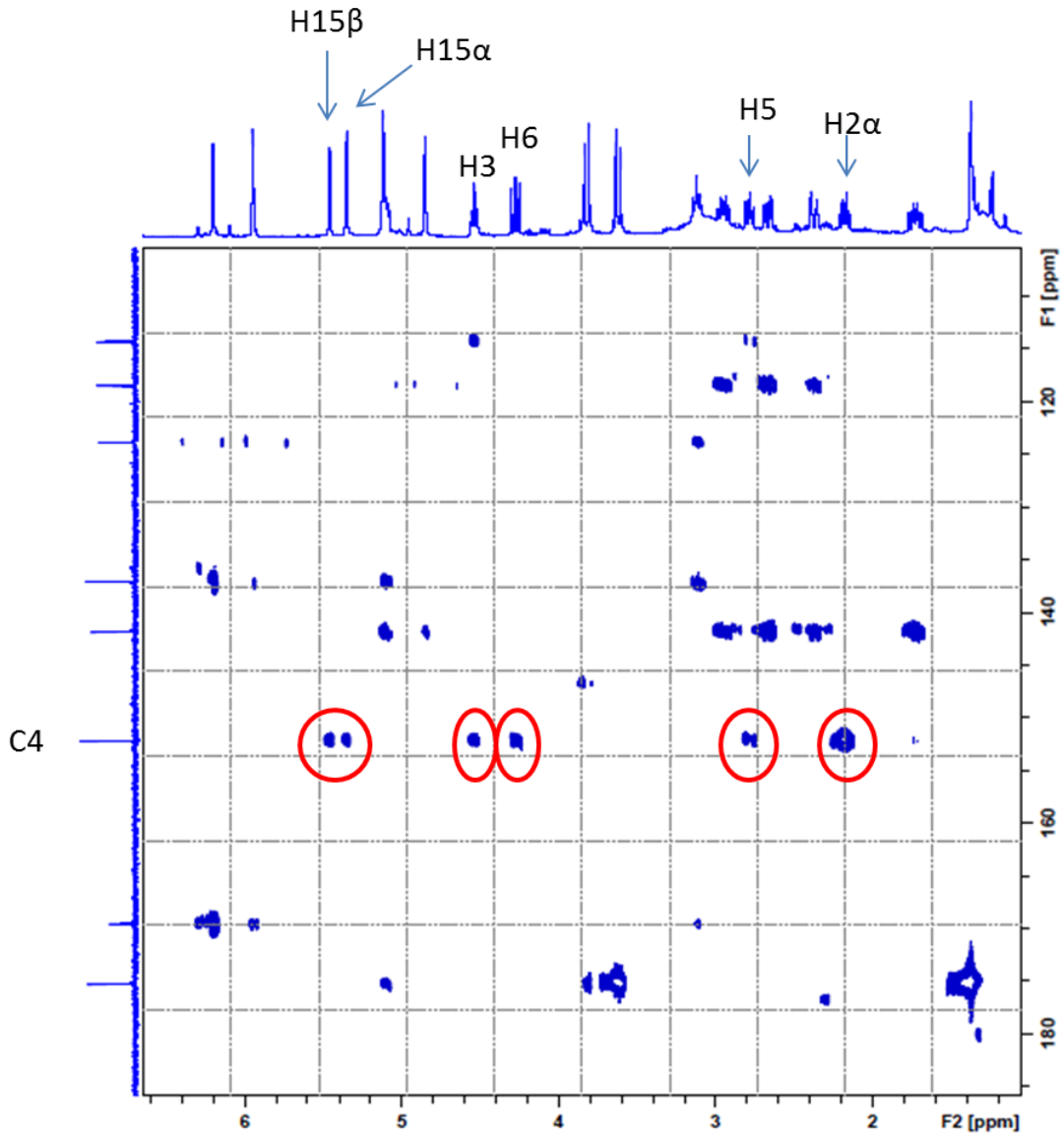

Figure 19. 2D NMR–HMBC spectrum ($CDCl_3$), for compound 7, with amplifications between 1-6.6 ppm (^1H) and 105-185 ppm (^{13}C).

A.5. Data retrieved from the spectra of ^{1}H NMR and ^{13}C NMR

Table 4. NMR data of compounds 6 and 6a. [a]

	6 [b]		6a [c]	
Position	**δ (^{1}H)**	**δ (^{13}C)**	**δ (^{1}H)**	**δ (^{13}C)**
1	3.22 *dd broad* (8.0, 8.1)	40.94	3.14 *ddd* (8.7, 4.8, 2.2)	39.60
2α	2.48 *dd* (11.8, 7.4)	42.70	2.31-2.37 *m*	47.46
2β	2.51 *dd* (11.8, 8.4)	-	2.51-2.58 *m*	-
3	-	218.32	-	217.80
4	2.44 *q broad* (9.7, 7.2)	46.96	2.53 *q* (7.7)	43.38
5	2.27-2.36 *m*	51.78	2.27-2.35 *m*	50.95
6	4.08 *dd* (9.7, 9.3)	80.81	4.05 *t* (9.7)	83.32
7	3.31 *dt* (9.9, 3.3)	46.88	2.65-2.67 *m*	44.94
8	5.13 *ddd* (9.9, 6.3, 5.7)	76.01	3.63 *ddd* (13.2, 9.6, 5.7)	73.91
9α	2.32 *dd* (13.6, 6.3)	41.11	2.19 *dd* (12.4, 9.6)	47.22
9β	2.88 *dd* (13.6, 5.7)	-	2.87 *dd* (12.4, 5.7)	-
10	-	141.59	-	144.13
11	-	135.65	2.51-2.58 *m*	38.64
12	-	169.12	-	174.99
13α	6.33 d (3.6)	125.74	2.27-2.35 *m*	54.53
13β	6.11 d (3.2)	-	2.27-2.35 *m*	-
14α	4.97 s	117.77	4.78 *s*	114.78
14β	5.1 s	-	5.08 *s*	-
15	1.26 d (7.3)	15.51	1.23 *d* (7.7)	14.54
$N(CH_3)_2$	-	-	2.65 *s*	34.85
1'	-	175.15	-	175.18
2'	-	76.12	-	73.91
3'α	3.88 d (11.6)	67.98	2.93 *dd* (12.9, 2.3)	59.17
3'β	3.65 d (11.6)	-	2.56 *dd* (12.9, 1.5)	-
4'	1.39 s	22.33	2.03 *s*	20.79

[a] δ (ppm) and J (Hz), when resolved, indicated in parentheses; Data were obtained in CDCl3; *s* singlet, *d* doublet, *m* multiplet, *t* triplet, *q* quartet, *dd* double doublet, *ddd* double doublet, *dt* double triplet; [b] obtained in the Bruker Avance III - 400 NMR; [c] obtained in the Bruker ASCEND - 700 NMR.

Table 5. NMR data of compounds 7 and 7a. [a]

	7 [b]		7a [c]	
Position	**δ (^{1}H)**	**δ (^{13}C)**	**δ (^{1}H)**	**δ (^{13}C)**
1	2.96 *ddd* (12.0, 8.2, 7.7)	45.71	2.92 *dd broad* (8.8, 7.6)	43.85
2α	2.18 *dd broad* (12.8, 7.6)	39.22	2.31 *ddd* (13.4, 7.4, 7.6)	38.57
2β	1.73 *ddd* (12.8/12.0/7.6	-	1.74 *ddd* (13.4, 8.8, 2.0)	-
3	4.54 *t broad* (7.6)	73.75	4.53 *dt* (7.4, 2.0)	73.68
4	-	152.16	-	152.61
5	2.78 *dd* (9.5, 8.2)	52	2.85-2.89 *m*	49.65
6	4.28 *dd* (9.5, 9.2)	78.22	4.10 *t* (9.9)	79.14
7	3.12 *dt* (9.2, 3.2)	47.09	2.68 *ddd* (11.4, 9.9, 2.6)	45.48
8	5.09 *m*	75.79	3.59-3.62 *m*	73.27
9α	2.67 *dd* (13.8, 3.9)	35.98	2.72 *dd* (13.1, 4.7)	43.23
9β	2.37 *d broad* (13.8)	-	2.20 *dd* (13.1, 7.7)	-
10	-	141.81	-	143.35
11	-	137.04	2.33-2.37 *m*	45.48
12	-	169.48	-	175.14
13α	6.20 *d* (3.6)	123.81	2.09 *d broad* (9.7)	57.77
13β	5.95 *d* (3.2)	-	2.06 *d broad* (9.7)	-
14α	4.85 *s*	118.43	5.04 *s*	116.07
14β	5.12 *s*	-	5.06 *s*	-
15 α	5.35 *s*	114.24	5.34 *s*	112.70
15β	5.46 *s*	-	5.39 *s*	-
$N(CH_3)_2$	-	-	2.66 *s*	34.84
1'	-	175.24	-	175.29
2'	-	76.2	-	73.68
3'α	3.82 *d* (11.6)	68.23	2.85-2.89 *m*	59.42
3'β	3.62 *d* (11.6)	-	2.55 *d* (12.4)	-
4'	1.38 *s*	22.09	2.04 *s*	20.80

[a] δ (ppm) and J (Hz), when resolved, indicated in parentheses; Data were obtained in CDCl3; *s* singlet, *d* doublet, *m* multiplet, *t* triplet, *q* quartet, *dd* double doublet, *ddd* double doublet, *dt* double triplet; [b] obtained in the Bruker Avance III - 400 NMR; [c] obtained in the Bruker ASCEND - 700 NMR.